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Deregulation Of The Hippo Pathway Suppresses Differentiation And Promotes Sarcomagenesis

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Abstract
Terminal differentiation opposes proliferation in the vast majority of tissue types. As a result, loss of lineage differentiation is a hallmark of aggressive cancers, including soft tissue sarcomas (STS). Consistent with these observations, undifferentiated pleomorphic sarcoma (UPS), an STS subtype devoid of lineage markers, is among the most aggressive and lethal sarcomas in adults. Though tissue-specific features are lost in these mesenchymal tumors they are most commonly diagnosed in skeletal muscle and are thought to develop from transformed muscle progenitor cells. We have found that a combination of HDAC (Vorinostat) and BET bromodomain (JQ1) inhibition partially restores differentiation to skeletal muscle UPS cells and tissues, enforcing a myoblast-like identity. Importantly, differentiation is contingent upon downregulation of the Hippo pathway transcriptional effector, Yes-activated protein 1 (YAP1) and its downstream effectors in the NF-κB pathway. YAP1 and NF-κB are critical mediators of myoblast proliferation and their activity must be down regulated to permit differentiation. Previously, we observed that Vorinostat/JQ1 suppresses YAP1 and NF-κB activity and as a result, inhibits tumorigenesis, and promotes differentiation. Here I show that YAP1 and NF-κB activity suppress circadian clock function, inhibiting differentiation and promoting unchecked proliferation. In most tissues clock activation is antagonized by the unfolded protein response (UPR). However, skeletal muscle differentiation requires both Clock and UPR, suggesting the molecular link between them is unique in muscle. In skeletal muscle-derived UPS we observed that YAP1 suppresses PERK and ATF6-mediated UPR target expression as well as clock genes. These pathways govern metabolic processes including autophagy and their disruption supports a shift in metabolism toward cancer cell-associated glycolysis and hyper-proliferation. Treatment with Vorinostat/JQ1 successfully inhibited glycolysis/MTOR signaling, activated the clock, and upregulated the UPR and autophagy via YAP1. These findings support the use of epigenetic modulators to treat human UPS and define the connection between these pathways and their effects on tissue differentiation. Additionally, we identify specific metabolic and differentiation genes as potential biomarkers of treatment efficacy.

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Adrian Rivera Reyes

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DEDICATORIA

¡Dedico mi tesis a mi familia! A mis padres, Hernán e Ivette, por la vida que me proveyeron y por encaminarme a un futuro exitoso en el cual mi educación y bienestar han sido lo primordial. Gracias por luchar para asegurar que tuviera mejores oportunidades que las que ustedes tuvieron. Gracias por enseñarme el valor del trabajo duro, por su apoyo y por creer en mí, incluso cuando nadie más lo hizo. ¡Gracias por sus sacrificios, sin su dedicación y esfuerzo constante este momento no sería posible! ¡Los amo!

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¡A todxs, los llevo en mi corazón!

DEDICATION

I dedicate my thesis to my family! To my parents, Hernán and Ivette, for the life you provided me and for guiding me towards a successful future, for which my education has always been the main focus. Thank you for fighting your entire life to ensure that I had better opportunities than the ones you had. Thank you for teaching me the value of hard work, for your support, and for believing in me, even when no one else did. Thank you for all your sacrifices, without your dedication and constant efforts this moment would not be possible! I love you!

To my brother, Hernán, for being my biggest role model. Ever since I was a kid, I wanted to be like you and do everything you did. You have always served me as inspiration and example to follow. Thank you for your unconditional support, help, and advice!

To my nephew, Diego, for teaching me what unconditional love is and for bringing me absolute happiness. I hope you see Tío's accomplishment as an example that, despite adversity, you too can accomplish all your goals and achieve your dreams. Diegolín, remember to do good, that nothing is impossible and that I'll always be here for you. Thank you for existing; go conquer the world!

I dedicate my thesis to my mentors! To my thesis advisor, Karin, for opening the doors to your lab when I needed it most and no one else did. Thank you for your advice, honesty, and mentorship. Thank you for investing in my future!

To my friend and mentor, Arnaldo, for motivating me to persist. Thank you for the opportunity to come to Penn. I acknowledge that opportunity was instrumental for my career. Thank you for always having the door to your office open, for your advice and support, and for your willingness to help others. Above all, thank you for the great work that you do and for makinour community welcoming to everyone.

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You'll always be in my heart!

ABSTRACT

DEREGULATION OF THE HIPPO PATHWAY SUPPRESSES DIFFERENTIATION AND PROMOTES SARCOMAGENESIS

Adrian Rivera Reyes

T.S. Karin Eisinger, Ph.D.

Terminal differentiation opposes proliferation in the vast majority of tissue types. As a result, loss of lineage differentiation is a hallmark of aggressive cancers, including soft tissue sarcomas (STS). Consistent with these observations, undifferentiated pleomorphic sarcoma (UPS), an STS subtype devoid of lineage markers, is among the most aggressive and lethal sarcomas in adults. Though tissue-specific features are lost in these mesenchymal tumors they are most commonly diagnosed in skeletal muscle and are thought to develop from transformed muscle progenitor cells. We have found that a combination of HDAC (Vorinostat) and BET bromodomain (JQ1) inhibition partially restores differentiation to skeletal muscle UPS cells and tissues, enforcing a myoblast-like identity. Importantly, differentiation is contingent upon downregulation of the Hippo pathway transcriptional effector, Yes-activated protein 1 (YAP1) and its downstream effectors in the NF-κB pathway. YAP1 and NF-κB are critical mediators of myoblast proliferation and their activity must be down regulated to permit differentiation. Previously, we observed that Vorinostat/JQ1 suppresses YAP1 and NF-κB activity and as a result, inhibits tumorigenesis, and promotes differentiation. Here I show that YAP1 and NF-κB activity suppress circadian clock function, inhibiting differentiation and promoting unchecked proliferation. In most tissues clock activation is antagonized by the unfolded protein response (UPR). However, skeletal muscle differentiation requires both Clock and UPR, suggesting the molecular link between them is unique in muscle. In skeletal muscle-derived UPS we observed that YAP1 suppresses PERK and ATF6-mediated UPR target expression as well as clock genes. These pathways govern metabolic

processes including autophagy and their disruption supports a shift in metabolism toward cancer cell-associated glycolysis and hyper-proliferation. Treatment with Vorinostat/JQ1 successfully inhibited glycolysis/MTOR signaling, activated the clock, and upregulated the UPR and autophagy via YAP1. These findings support the use of epigenetic modulators to treat human UPS and define the connection between these pathways and their effects on tissue differentiation. Additionally, we identify specific metabolic and differentiation genes as potential biomarkers of treatment efficacy.

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Chapter 1: Introduction

Soft Tissue Sarcoma (STS) Overview

Nearly 200,000 people worldwide and 12,000 Americans are diagnosed with soft-tissue sarcoma (STS) with approximately 4,000 deaths every year in the United States (Taylor et al. 2011; Siegel et al. 2018). This complex set of tumors arises in mesenchymal tissues such as connective, adipose tissue, and skeletal muscle tissues (Jo & Fletcher 2014). STS are rare adult tumors that account for less than 1% of all adult malignancies (Siegel et al. 2018). The roughly 65 known sarcoma subtypes can be very generally divided into two groups 1) majority pediatric sarcomas which most frequently develop as a result of chromosomal translocations and have near diploid karyotypes (Borden et al. 2003; Mertens et al. 2009; Kadoch & Crabtree 2013; Ciarapica et al. 2014; Shern et al. 2014) and 2) adult sarcomas that have no known consistent oncogenic mutations, but ~50% of which bear mutations in tumor suppressor genes like RB1 or p53, often referred to as genetically and karyotypically complex (Borden et al. 2003; Mertens et al. 2010; W. Lee et al. 2014; De Raedt et al. 2014; M. Zhang et al. 2014). Due to their karyotype complexity, variety of subtypes, and the lack of known drivers, adult sarcomas are poorly understood. Thus, treatment options are generally limited to radiation and surgery, as inadequate characterization has precluded the development of targeted therapies (Taylor et al. 2011; Pappo et al. 2014; Wagner et al. 2015).

Sarcomas with simple genetic alterations generally arise because of gene translocations. In some cases, there is only one defining genetic abnormality present during tumor initiation (Mertens et al. 2009). These tumors with near diploid karyotypes account for one third of all sarcomas (Mertens et al. 2009). In contrast, genetically and karyotypically complex sarcomas can develop slowly and accumulate mutations or aberrations that lead to genetic complexity throughout time (Horvai et al. 2009; Rosai et al. 1996; Snyder et al. 2009). Most high-grade karyotypically complex sarcomas arise spontaneously and without previous lower-grade lesions

(Helman & Meltzer 2003; Clark et al. 2005; Mertens et al. 2010). These tumors are poorly understood due to their complexity, variety of subtype, and lack of known drivers. Current treatment options are limited to radiation and surgery, because development of targeted therapies has lagged due to inadequate characterization of STS (Pappo et al. 2014; Wagner et al. 2015; Taylor et al. 2011). Our current work focuses on an aggressive adult tumor found in skeletal muscle, undifferentiated pleomorphic sarcoma (UPS).

Undifferentiated Pleomorphic Sarcoma (UPS): an STS Subtype

Undifferentiated pleomorphic sarcoma (UPS) is one of the most common and frequently diagnosed adult STS subtypes (Jo & Fletcher 2014). Because UPS's etiology is unknown, it is a diagnosis of exclusion (Lawrence et al. 1987; Jo & Fletcher 2014). UPS tumors are characterized by cytogenetic abnormalities such as chromosomal deletions, amplifications, chromosomal gains and losses, and aneuploidy (Albertson et al. 2003). These high-grade tumors do not seem to present consistent genetic aberrations or chromosomal abnormalities, which makes it difficult to identify the contributions of any genetic abnormality in these tumors (Mertens et al. 2002). Although UPS can be found in various tissues, it is most commonly found in skeletal muscle (Ballinger et al. 2016).

Currently, there are two models regarding the etiology of UPS. The first model suggests that UPS represents not just a single sarcoma subtype, but a number of phenotypes that are common to other sarcomas in advanced stages (Matushansky et al. 2009). In this model, the common idea is that tumors de-differentiate as they progress, resulting in a high-grade UPS (Matushansky et al. 2009; Murphey 2007). The second model suggests that UPS tumors result from the transformation of mesenchymal stem cells (Matushansky et al. 2009; Murphey 2007). In this model, it is thought that muscle progenitor cells, or satellite cells, give rise to skeletal muscle-derived UPS (Rubin et al. 2011).

UPS tumors can arise in any part of the body, but they most often develop in extremities and torso (Lawrence et al. 1987; Pitcher et al. 1994; Zornig et al. 1992). The majority of UPS tumors present themselves as painless masses throughout the body and grow locally (Jo & Fletcher 2014). These tumors are associated with poor clinical outcome, as they frequently enter the bloodstream and metastasize to the lungs (Christie-Large et al. 2008; Matushansky et al. 2009). Thus, understanding the cellular and molecular mechanisms that direct and control UPS metastasis is an important and prominent field of research. The tumor microenvironment in UPS has been shown to be dependent on lack of oxygen or hypoxia (Eisinger-Mathason et al. 2013).

Due to its metastatic capacity and lack of targeted therapies, UPS is particularly hard to treat (Ballinger et al. 2016). Targeted therapies for UPS have proven difficult because of the lack of evidence and knowledge regarding the molecular mechanisms that drive these tumors (Taylor et al. 2011; Kelleher & Viterbo 2013). Among the treatments that exist for UPS patients are surgery, radiation, and chemotherapy (Taylor et al. 2011). The most effective treatment includes radical surgeries, in which the tumor, blood vessels and lymph nodes are removed (Linehan et al. 2000). Conversely, as radical surgeries are not always an option for patients, more conservative surgeries are performed, which can often lead to tumor recurrence. In order to improve patient outcome, prognosis and quality of life, these conservative surgeries could be combined with targeted therapies that inhibit cell proliferation or metastasis potential.

In order to develop new treatments or targeted therapies for UPS, it is essential to develop accurate models. A common model used to understand UPS tumors is the *LSL Kras*^{G12D}*; Trp53*$^{-/-}$ (KP) mouse model. This model of UPS skeletal muscle UPS relies simultaneous Cre-dependent expression which is injected into the right gastrocnemius muscle of mice in order to activate oncogenic Kras and delete the p53 gene (Kirsch et al. 2007). Tumors arise approximately 45 days post-intramuscular injection of adenovirus expressing Cre recombinase. Although human sarcomas do not frequently express mutated *KRAS*, KP sarcomas of

gastrocnemius muscle recapitulate human UPS with regard to transcriptional profiles, histology, morphology, and metastatic capacity (Mito et al. 2009). Importantly, roughly 50% of human sarcomas possess *Trp53* mutations, suggesting that loss of p53 function combined with hyper-proliferation induced errors will initiate UPS (Guijarro et al. 2013). This hypothesis is supported by additional models in which KRAS mutation can be replaced by intramuscular cardiotoxin injection and still develop UPS tumors (Van Mater et al. 2015). Moreover, the tumors that develop from the KP model are driven by hypoxia and HIF1α to metastasize to the lung, as does human UPS (Eisinger-Mathason et al. 2013). Additionally, within weeks of injection, subcutaneous allografts of murine UPS cells will also metastasize to the lung (Eisinger-Mathason et al. 2013). This murine model along with in vitro and human UPS tumor sections allow for the investigation of molecular mechanisms that promote primary UPS formation and metastasis.

Skeletal Muscle Myogenesis and Differentiation

As mentioned earlier, one hypothesis for UPS tumor formation suggests that these tumors result from the transformation of mesenchymal stem cells, or skeletal muscle progenitor cells (Matushansky et al. 2009; Murphey 2007; Rubin et al. 2011). Skeletal muscle myogenesis is a complex process that commences during embryonal development and by which muscle tissues are formed (Parker et al. 2003; Sambasivan & Tajbakhsh 2007). During embryonic development, mesenchymal stem cells give rise to muscle fibers. Next, mesenchymal stem cells give rise to muscle fibers that fuse with myoblasts to form multinucleated myotubes (Parker et al. 2003; Sambasivan & Tajbakhsh 2007). Muscle progenitor cells then enter a quiescent state and reside as satellite cells (Schultz 1996; Davis & Fiorotto 2009). To maintain tissue homeostasis, skeletal muscle tissue relies on satellite cells that can self-renew muscle tissue by differentiating into new fibers (Schmalbruch & Lewis 2000; Pellettieri & Sánchez Alvarado 2007; Chargé & Rudnicki 2004). Thus, it is hypothesized that deregulation of satellite cell differentiation contributes to UPS formation.

Conversely, myogenesis is regulated by cell surface receptors that activate intracellular signal transduction pathways (Kuang et al. 2008; Bentzinger et al. 2010). These signaling molecules promote transcription of genes and microRNAs that allows precursor cells to commit to the myogenic lineage and develop into muscle (He et al. 2009). In the embryo, the first skeletal muscle cells that form are myocytes (Lyons, Buckingham, et al. 1991; Lyons, Mühlebach, et al. 1991; Chal et al. 2018). These newly formed myocytes further differentiate via Wnt11 expression and signaling, which allows for their elongation along the anterior-posterior axis (Denetclaw et al. 1997; Gros et al. 2009). Next, the myocytes mature into the myotome once they express MyoD and Myf5 (Sassoon et al. 1988; Kiefer & Hauschka 2001). Once both MyoD and Myf5 are expressed, the myocytes commit to the muscle lineage and develop into muscle (Pownall et al. 2002). MYOD is a powerful and tightly regulated muscle transcription factor, which along with myocyte enhancer factors (MEFs) drive a muscle differentiation transcriptional program (Pownall et al. 2002; Conerly et al. 2016). Moreover, there is evidence demonstrating that the Hippo signaling pathway modulates myogenesis and muscle regeneration, as well as inhibit embryonic stem cell differentiation (Fischer et al. 2016; Lian et al. 2010).

Hippo Signaling Pathway and Muscle Differentiation

The Hippo signaling pathway is an evolutionary conserved network first discovered and characterized in Drosophila melanogaster and is the master regulator of organ size control, cell proliferation, cell fate, stem cell self-renewal, and tumorigenesis (Misra & Irvine 2018; Staley & Irvine 2012; Ramos & Camargo 2012). The Hippo signaling pathway is a kinase cascade that responds to various external stimuli and key regulators (Hamaratoglu et al. 2006; Yu & Guan 2013). The pathway consists of the mammalian kinases MST1/2 and LATS1/2, the effector protein Yes-activated protein (YAP1) and the transcriptional co-activator with PDZ binding motif (TAZ) (Yu & Guan 2013; Callus et al. 2006; Barron & Kagey 2014). For the purpose of this dissertation, I will focus solely on YAP1 and not TAZ. The MST1/2 kinases directly phosphorylate LATS1/2 and the protein SAV1 facilitates the interaction between both sets of proteins and

allowing for phosphorylation to occur (Yu & Guan 2013; Callus et al. 2006). In order to bind the DNA and drive the Hippo transcriptional program, YAP1 must form a heterodimer with the Tea Domain Family transcription factor (TEAD). The TEAD proteins contain a canonical M-CAT motif (5'-TCATTCCT-3') that is often found in the promoters of muscle associated genes (Larkin et al. 1996; Jiang et al. 2000). YAP1-TEAD complexes bind the M-CAT motif to drive or inhibit transcription of genes (Ye et al. 2018; Eisinger-Mathason et al. 2015; Liu-Chittenden et al. 2012; Zhao et al. 2007; Barron & Kagey 2014).

In order for YAP1 to translocate into the nucleus, bind TEAD, and promote its transcriptional program, YAP1 must remain unphosphorylated (Ren et al. 2010). The LATS1/2 kinases mediate YAP1 phosphorylation (Zhao et al. 2007). Phosphorylation of the YAP1 hinders its ability to translocate into the nucleus and is thus sequestered via 14-3-3 binding and degraded in the cytoplasm (Zhao et al. 2007) **(Figure 1)**. As a transcriptional modulator, YAP1 is able to regulate expression of genes that are involved in tumor cell proliferation, epithelial to mesenchymal transition, invasion and metastasis (Zhao et al. 2007; Eisinger-Mathason et al. 2015; Mizuno et al. 2012; Yang et al. 2015; Muramatsu et al. 2011).

The Hippo pathway plays an important role in modulating myogenesis and muscle regeneration, and also inhibits embryonic stem cell differentiation (Fischer et al. 2016; Lian et al. 2010). During skeletal muscle myogenesis, activated satellite cells expand and differentiate, whereas quiescent satellite cells localize between the basal lamina and plasma membrane of myofibers (M. Zhang & McLennan 1999; Lepper & Fan 2010). In satellite cell-derived myoblasts, it has been shown that YAP1 knockdown does not affect differentiation but reduces proliferation (Nagata et al. 2006). There is evidence showing that YAP1 activity inhibits skeletal muscle differentiation, as YAP1 overexpression leads to inhibition of MYOD expression (Gee et al. 2011; Watt et al. 2015). Moreover, in vitro overexpression of constitutively active YAP1 in myoblast precursors inhibits differentiation by altering the transcriptional program (Ishibashi et al. 2005; De

Falco & De Luca 2006; Watt et al. 2010). In terms of YAP1 regulation, it has been demonstrated that caspase 3 activates the Hippo kinase, MST1, during myoblast differentiation (Fernando et al. 2002).

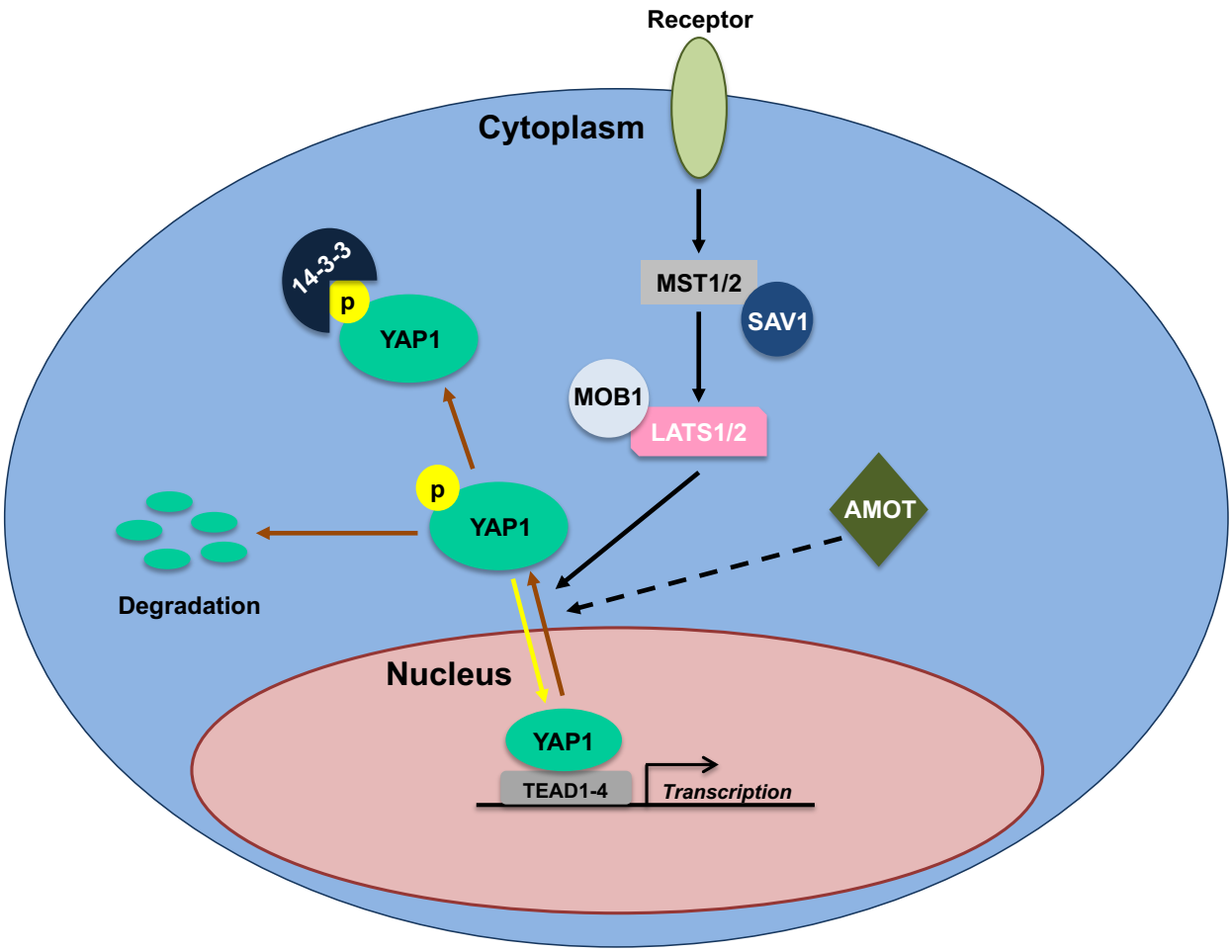


Figure 1: Hippo signaling pathway

Last, deregulation of YAP has been observed in epithelial tumors such as breast and liver cancer, among others, as well as mesenchymal tumors, such as soft tissue sarcoma (Eisinger-Mathason et al. 2015; Muramatsu et al. 2011; Z. Wang et al. 2014; M. Z. Xu et al. 2009; M. Song et al. 2012; Ahmed et al. 2015; Mohamed et al. 2015). Research from our lab has shown that the Hippo signaling pathway is silenced in UPS, which allows for uncontrolled activation of YAP1 (Eisinger-Mathason et al. 2015; Mizuno et al. 2012). YAP1 is also stabilized in human UPS tumors and promotes a pro-proliferation transcriptional program (Eisinger-Mathason et al. 2015; Mizuno et al. 2012). YAP1 stabilization in these tumors and other sarcomas is due to Hippo pathway kinase deletion and epigenetic silencing of its inhibitor, Angiomotin (AM(Ye & Eisinger-Mathason 2016). These perturbations stabilize YAP1 at the protein level and enhance its transcriptional activity by increasing its nuclear localization (Ye & Eisinger-Mathason 2016). Our work has also shown that elevated YAP1 levels result in heightened NF-κB signaling in UPS (Ye & Eisinger-Mathason 2016). Interestingly, in myoblasts, it has been shown that NF-κB signaling downregulates MYOD expression in myoblast seem to be mutually exclusive (Guttridge et al. 2000). Though well studied in epithelial tumors, the specific downstream effectors of YAP1 in sarcomas are still being elucidated.

NF-κB Signaling Pathway and Muscle Differentiation

The NF-κB signaling hub is a family of transcription factor regulators that consists of DNA binding proteins (Karin 2006; Perkins 2012). The NF-κB family of proteins are known to regulate cell proliferation, inhibit apoptosis, promote cell migration and invasion, and stimulate angiogenesis and metastasis (Karin & Greten 2005). NF-κB activation can be induced by viral and bacterial infections, necrotic cell products, DNA damage, oxidative stress, and proinflammatory cytokines (Karin & Greten 2005). The two pathways that comprise the NF-κB signaling hub are the canonical and noncanonical pathways (Karin & Greten 2005) **(Figure 2)**.

The canonical pathway is activated transiently and rapidly by proinflammatory cytokines that interact with specific receptors or adaptor molecules (Karin & Greten 2005).

The effector molecules for the canonical pathway are p50-p65 (RELA) heterodimers (Karin & Greten 2005; Sun 2017; Bonizzi & Karin 2004). In canonical NF-κB, the activation of the p50-p65 dimer depends on the degradation of the NF-κB specific inhibitor IκB, which releases the dimer after it becomes phosphorylated by the IKK subunits, IKKα and IKKβ (Karin & Greten 2005). The IKK subunits are necessary to activate both NF-κB pathways. The IKKα-IKKβ heterodimer activates the canonical pathway (Karin & Greten 2005; Sun 2017; Bonizzi & Karin 2004; Senftleben et al. 2001). Additionally, canonical signaling requires the IKKγ regulatory subunit, or NEMO, which holds the IKKα-IKKβ heterodimer in place so that the heterodimer can trans-autophosphorylate (Karin & Greten 2005; Karin 2006; G. Xu et al. 2011; Rothwarf & Karin 1999). In order for the trans phosphorylation of the IKK heterodimer to occur, the IKKγ subunit must be polyubiquitinated in its K63 residue (Chen 2012; Tokunaga & Iwai 2012) **(Figure 2, left)**. NF-κB activity is also modulated downstream by various microRNAs (miRNAs) that target mRNAs coding for the IKK proteins, as well as it can be modulated upstream by other protein regulators (X. Ma et al. 2011; Boldin & Baltimore 2012; Taganov et al. 2006).

Furthermore, the noncanonical pathway activation is a slower process that requires de novo synthesis of NF-κB inducing kinase (NIK) (Vallabhapurapu & Karin 2009). NIK phosphorylates the IKKα-IKKα homodimer, which is required to activate the noncanonical pathway (Karin & Greten 2005; Sun 2017; Bonizzi & Karin 2004; Senftleben et al. 2001). Next, the IKKα–IKKα homodimer phosphorylates p100, which is then polyubiquitinated and cleaved to produce p52 (Karin & Greten 2005; Sun 2017; Bonizzi & Karin 2004; Senftleben et al. 2001). The effector molecules for the noncanonical pathway are p52 and RELB, which form a heterodimer and translocate into the nucleus to drive or inhibit transcription of target genes (Karin & Greten 2005; Sun 2017; Bonizzi &

Karin 2004) **(Figure 2, right)**. For the purpose of this dissertation, I will focus on canonical NF-κB signaling.

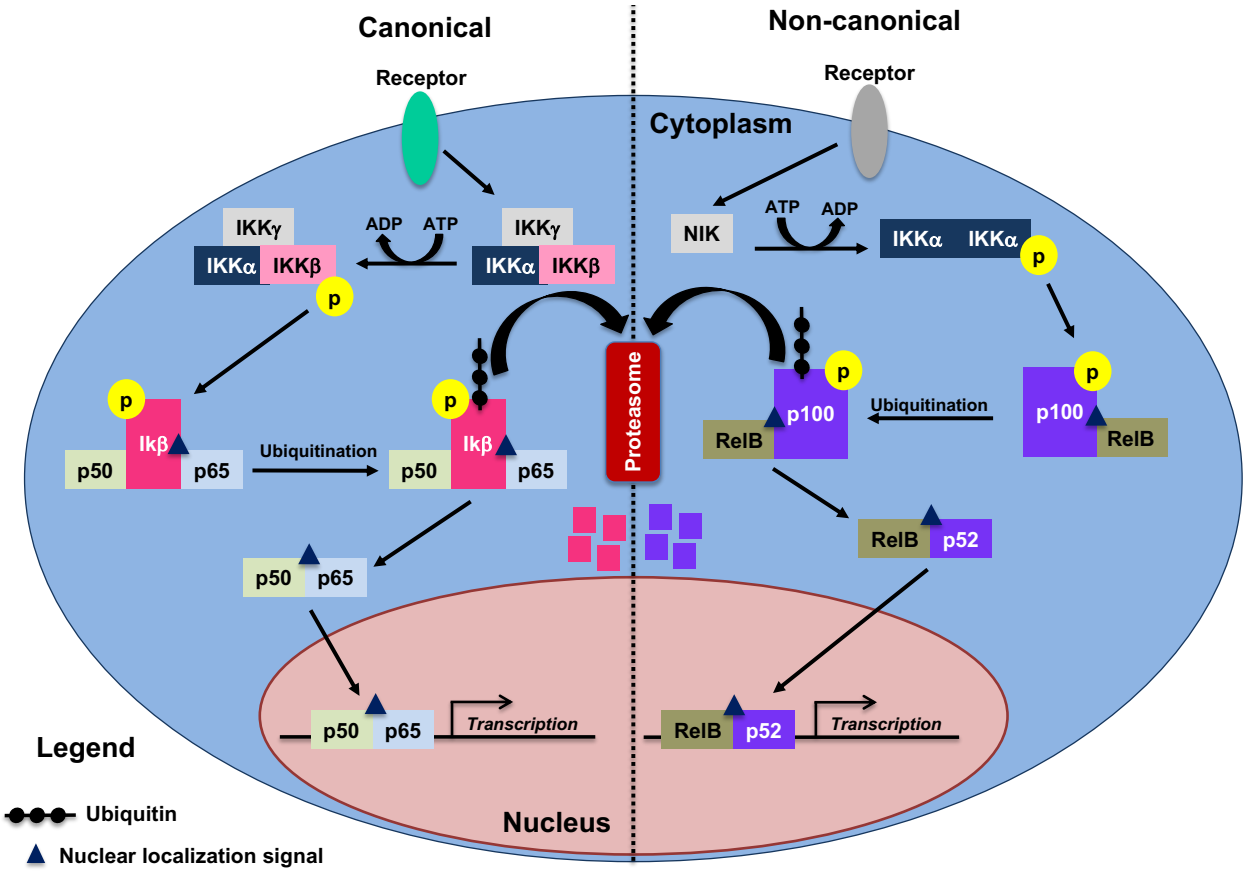


Figure 2: Canonical (left) and non-canonical (right) NF-κB pathways

NF-κB signaling activation has been associated with promoting myogenesis formation but also its hyperactivation has been associated with multiple skeletal muscle abnormalities such as a cachexia, muscular dystrophy, inflammatory myopathy and rhabodomyosarcomas, among others (Creus et al. 2009; Li et al. 2008; Mourkioti & Rosenthal 2008; Peterson & Guttridge 2008). Additionally, NF-κB transcriptional and DNA binding activity has been shown to decline over the course of myogenic differentiation (Egerman & Glass 2014; Catani et al. 2004; Dee et al. 2003; Guttridge et al. 2000). NF-κB signaling can also promote transcription of targets that repress myogenic differentiation, such as Cyclin D1, as well as by suppressing MyoD synthesis (Guttridge et al. 2000; Skapek et al. 1995; Sitcheran et al. 2003).

In cancer, NF-κB is often found constitutively active, and this hyperactivation is seldom due to genetic alterations (Karin & Greten 2005; Staudt 2010; Ben-Neriah & Karin 2011). In solid tumors, increased NF-κB activity is due to elevated production of activating cytokines (Ben-Neriah & Karin 2011; DiDonato et al. 2012). In human UPS, we have shown that NF-κB is the most transcriptionally active pathway (Ye et al. 2018). Additionally, we have demonstrated that YAP1 suppresses expression of a newly identified negative regulator of NF-κB signaling, Ubiquitin Specific Peptidase 31 (USP31), which predictably results in tumorigenesis and cell hyperproliferation (Ye et al. 2018; Tzimas et al. 2006; Lockhart et al. 2004). Additionally, we have established a connection between the Hippo signaling pathway effector protein, YAP1, and NF-κB signaling, as YAP1 inhibition restored UPS31 expression (Ye et al. 2018). Our findings suggest that skeletal muscle-derived UPS behave similar to proliferating myoblasts, as these tumors are not able to undergo differentiation due to persistent YAP1 and NF-κB activation. Interestingly, we and others have observed NF-κB signaling oscillation over time in skeletal muscle precursor cells as well as other tissues (Ye et al. 2018; Zambrano et al. 2016; X. Wang et al. 2015) (Ye et al., 2018; Zambrano et al., 2016; Wang et al., 2015). The oscillatory pattern exhibited by NF-κB signaling is reminiscent of circadian clock oscillation, which is known to

regulate NF-kB signaling and has been linked to muscle differentiation and normal myoblast proliferation (X. Wang et al. 2015; Andrews et al. 2010; McCarthy et al. 2007; X. Zhang et al. 2009).

Circadian Clock and Muscle Differentiation

The circadian clock is a 24-hour intrinsic molecular signaling network that synchronizes physiology and behavior and is known to regulate cellular metabolism (Lowrey & Takahashi 2004; Bass & Takahashi 2010; Altman et al. 2015). The clock is regulated by a network of positive and negative feedback loops that drive rhythmic expression of genes over a 24-hour period (Bell-Pedersen et al. 2005). The clock also regulates proliferation by controlling a diverse array of metabolic processes (Lowrey & Takahashi 2004; Bass & Takahashi 2010; Huang et al. 2016). The clock can also be influenced by environmental changes which in turn can affect gene expression and behavior (Lowrey & Takahashi 2004).

The main clock transcriptional proteins are CLOCK and BMAL1, which heterodimerize in the nucleus to promote transcription of clock target genes by binding to the E-box in the promoters of such genes (Lowrey & Takahashi 2004; Ripperger & Schibler 2006). The CLOCK-BMAL1 heterodimer promotes transcription of PERIOD (PER) and CRYPTOCHROME (CRY) genes, which are part of negative feedback loop that inhibits the CLOCK-BMAL1 heterodimer (Lowrey & Takahashi 2004; Ripperger & Schibler 2006). Once PER and CRY are expressed, they shuttle to the cytoplasm where they form a multimeric complex with casein kinase 1ε and translocate into the nucleus to inhibit the CLOCK-BMAL1 heterodimer and shut off circadian clock activity (Eide et al. 2005; Kume et al. 1999; C. Lee et al. 2001; Sangoram et al. 1998; Lowrey & Takahashi 2004; Ripperger & Schibler 2006). Importantly, PER is responsible for regulating and coordinating the entry of the complex into the nucleus (C. Lee et al. 2001). On the other hand, CRY is responsible for inhibiting the histone acetyltransferase p300, which decreases CLOCK-BMAL1-mediated transcription (Etchegaray et al. 2003) **(Figure 3)**. This PER-CRY-mediated feedback loop coupled

with the CLOCK-BMAL1-mediated feedforward loop give the clock the oscillatory pattern that characterizes it.

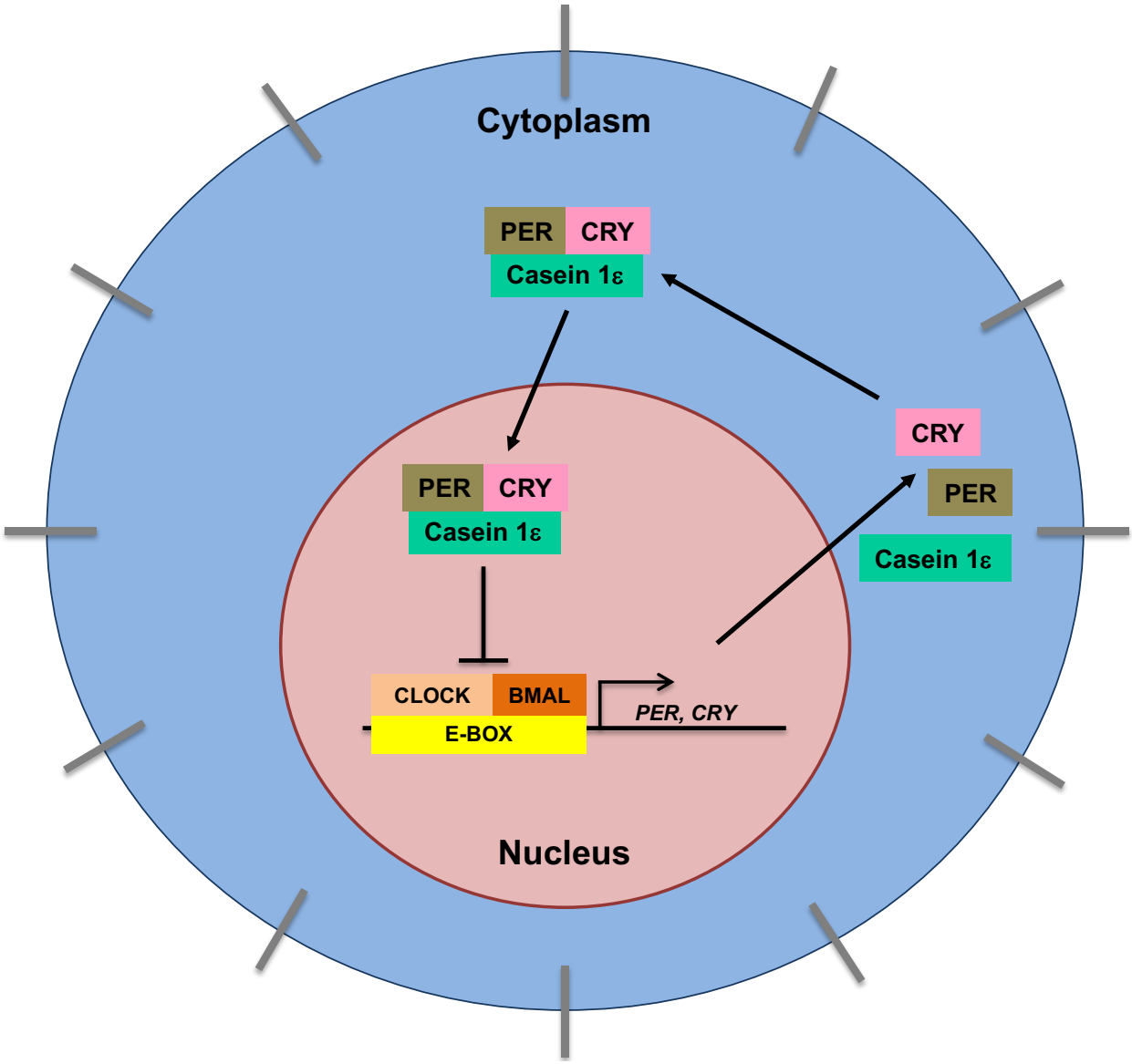


Figure 3: Circadian clock signaling

Circadian clock function has been shown to be necessary for muscle generation and differentiation, especially because MyoD is a transcriptional target of the clock (Andrews et al. 2010; X. Zhang et al. 2012; Lefta et al. 2011). Similar to NF-κB deficient mice, clock deficient mice have altered metabolism and develop muscle weakness and cachexia (Bohnert et al. 2016). Moreover, the clock has been shown to control NF-κB through induction of CLOCK (Spengler et al. 2012). Although there is some evidence showing a molecular connection between the clock and NF-κB signaling, the dynamics of how both signaling hubs regulate each other remain to be elucidated.

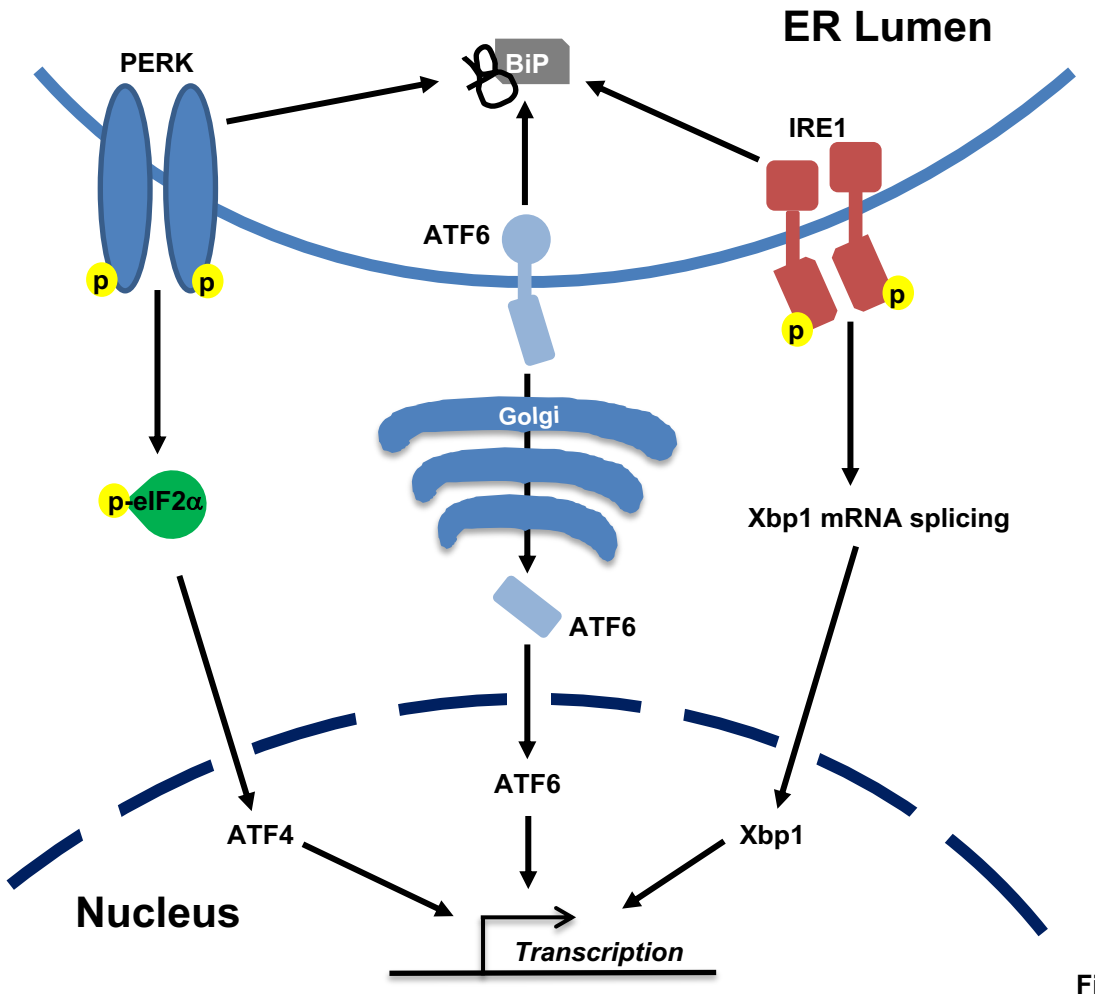
Recent studies have also shown that there is a molecular connection between the circadian clock and other pathways associated with muscle differentiation, such as the Unfolded Protein Response (UPR) and autophagy, which I will describe in further detail in the following sections (Bu et al. 2018; Koyanagi et al. 2011; Igarashi et al. 2007; Huang et al. 2016; Fortini et al. 2016; W.-S. Lee et al. 2015; Masiero et al. 2009). Moreover, disruption of circadian oscillation has been shown to promote tumorigenesis in different cancer settings (S. Wu et al. 2016; Ha et al. 2016; Fu et al. 2002).

Endoplasmic Reticulum (ER) Stress, the Unfolded Protein Response (UPR), and Muscle Differentiation

The UPR is a signaling hub that is mediated by the three ER transmembrane receptors: RNA-dependent protein kinase-like ER eukaryotic translation initiation factor 2 alpha kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6) (Hetz 2012; M. Wang & Kaufman 2014; J. Wu & Kaufman 2006). The ER is membrane bound organelle composed of a network of branching tubules arranged in sacs that is responsible for synthesis, folding, processing and trafficking proteins (Isler et al. 2005; Pyrko et al. 2007; Welihinda et al. 1999). ER stress is caused by different stimuli, such as protein misfolding, viral infections, imbalances in calcium levels, among others that may disrupt cell homeostasis, and can activate

the UPR via PERK, IRE1 and ATF6 receptors (Isler et al. 2005; Pyrko et al. 2007; Welihinda et al. 1999).

Prior to ER stress, the BiP/glucose-regulating protein 78 (GRP78) is bound to the UPR transmembrane receptors and renders them inactive (Harding et al. 1999; Hetz 2012; Y. Ma et al. 2002). Upon ER stress, BiP is released and associates with misfolded proteins in the ER lumen. Once BiP is released, PERK is autophosphorylated and starts a signaling cascade by phosphorylating the eukaryotic translation initiation factor 2a (eIF2a) and subsequent translation of activating transcription factor 4 (ATF4), which in turn induces transcription of DNA damage-inducible transcript 3 (*Ddit3*), which encodes C/EBP homologous protein (CHOP) (Harding et al. 1999; Hetz 2012; Y. Ma et al. 2002). Once ER stress has been relived, the PERK pathway is involved in the termination of UPR signaling by activating GADD34 (Ron & Walter 2007) **(Figure 4, left)**. IRE1, another UPR transmembrane receptor, becomes activated during ER stress by autophosphorylation (Flamment et al. 2012). IRE1 promotes splicing of XBP1 mRNA, which in turn increases the amount of ER chaperones and other components, in order to increase the protein folding capacity of the ER (Tirasophon et al. 1998) **(Figure 4, center)**. The third and last UPR transmembrane receptor is ATF6, which is cleaved and moves from the ER to the Golgi apparatus (Haze et al. 1999) (. The N-terminal of the cleaved ATF6 receptor translocates into the nucleus and in combination with spliced XBP1, increases proteins that alleviate ER stress (Flamment et al. 2012) **(Figure 4, right)**. Once all three pathways are active, they alleviate ER stress by regulating gene expression and protein synthesis to increase the protein folding capacity of the cell (Harding et al. 1999; Hetz 2012; Y. Ma et al. 2002).



Figur

Figure 4: Unfolded protein response (UPR) signaling pathways

Recent studies have shown that ER stress-induced UPR is necessary for muscle regeneration and muscle mass maintenance (Bohnert et al. 2018; Xiong et al. 2017; Bohnert et al. 2016). The PERK and ATF6 translated protein, CHOP, is known to stall differentiation by repressing MyoD in order to prevent premature differentiation of muscle progenitor stem cells (Alter & Bengal 2011). The PERK arm of the UPR has been shown to promote myofiber formation and is potentially required for maintaining satellite cells in a quiescent state in adult skeletal muscle (Xiong et al. 2017; Zismanov et al. 2016). Moreover, the ATF6 arm of the UPR is known to promote apoptosis in myoblasts that may be susceptible to cellular stress during differentiation (Nakanishi et al. 2005; Nakanishi et al. 2007). Although there is evidence of the UPR's contribution to skeletal muscle myogenesis and differentiation, the molecular mechanisms by which the UPR affects skeletal muscle differentiation remain to be elucidated.

The UPR and circadian clock have been shown to exhibit significant crosstalk in various tissues and cancer types (Bu et al. 2018). In skeletal muscle and muscle-derived tumors both the UPR and clock can be simultaneously upregulated and are important for differentiation (Andrews et al. 2010; Xiong et al. 2017; Bohnert et al. 2016). Recently, the UPR was identified to directly regulate the clock by suppressing the BMAL1-CLOCK heterodimer (Bu et al. 2018). Specifically, in vitro studies show that the PERK pathway triggers a 10-hour shift in circadian oscillation by inducing expression of miR-211, which directly suppresses BMAL1 and CLOCK (Bu et al. 2018). The clock has also been shown to control ER stress in order to mediate liver senescence through expression of Pdia3 (Yuan et al. 2017). Moreover, it was also shown that inhibition of the PERK/ATF4 pathway allowed for malignant progression of various cancers (Hart et al. 2012; Huber et al. 2016; Qing et al. 2012). Whereas Per1 is regulated by the IRE1 pathway, Per2 is a transcriptional target of ATF4 (Pluquet et al. 2013; Koyanagi et al. 2011). Thus, the PERK/PER2 axis could play an important role in preventing tumor initiation and progression.

Another critical function of the UPR and the clock is the regulation of metabolic processes (Bohnert et al. 2018). Activation of ATF4 is necessary for glutathione synthesis, whereas

activation of IRE1 controls hepatic lipid metabolism (Igarashi et al. 2007; Cretenet et al. 2010). Thus, when the UPR and clock are hijacked there is a possibility that both pathways are helping support a shift in metabolism that allows for tumor formation. Additionally, there is significant crosstalk between the UPR and autophagy, an important catabolic signaling process that promotes cell survival (White 2015). This relationship will be discussed further in the next section.

Autophagy and Muscle Differentiation

Autophagy is a natural self-degradation and catabolic mechanism important for balancing cellular energy sources that promotes degradation or recycling of cellular components (Senft & Ronai 2015; Glick et al. 2010). Autophagy is known to preserve organelle function, prevent toxic buildup of cellular waste products, and provides substrates to sustain metabolism during starvation (White 2015). There are three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (Glick et al. 2010). For the purpose of this dissertation I will focus on macroautophagy, referred to as autophagy from here on.

The autophagic machinery begins with the phagophore, an isolation membrane thought to arise from the ER membrane, the trans-Golgi, or endosomes (Axe et al. 2008; Simonsen & Tooze 2009). The phagophore then becomes the autophagosome by engulfing intracellular proteins, organelles, and ribosomes (Mizushima 2007). Next, the autophagosome fuses with the lysosome to form the autophagolysosome, which contains lysosomal proteases that degrade its cargo (Mizushima 2007; Glick et al. 2010) **(Figure 5)**. Last, nutrients and degradation products such as amino acids, are recycled and transported back into the cytoplasm by lysosomal permeases and transporters (Mizushima 2007).

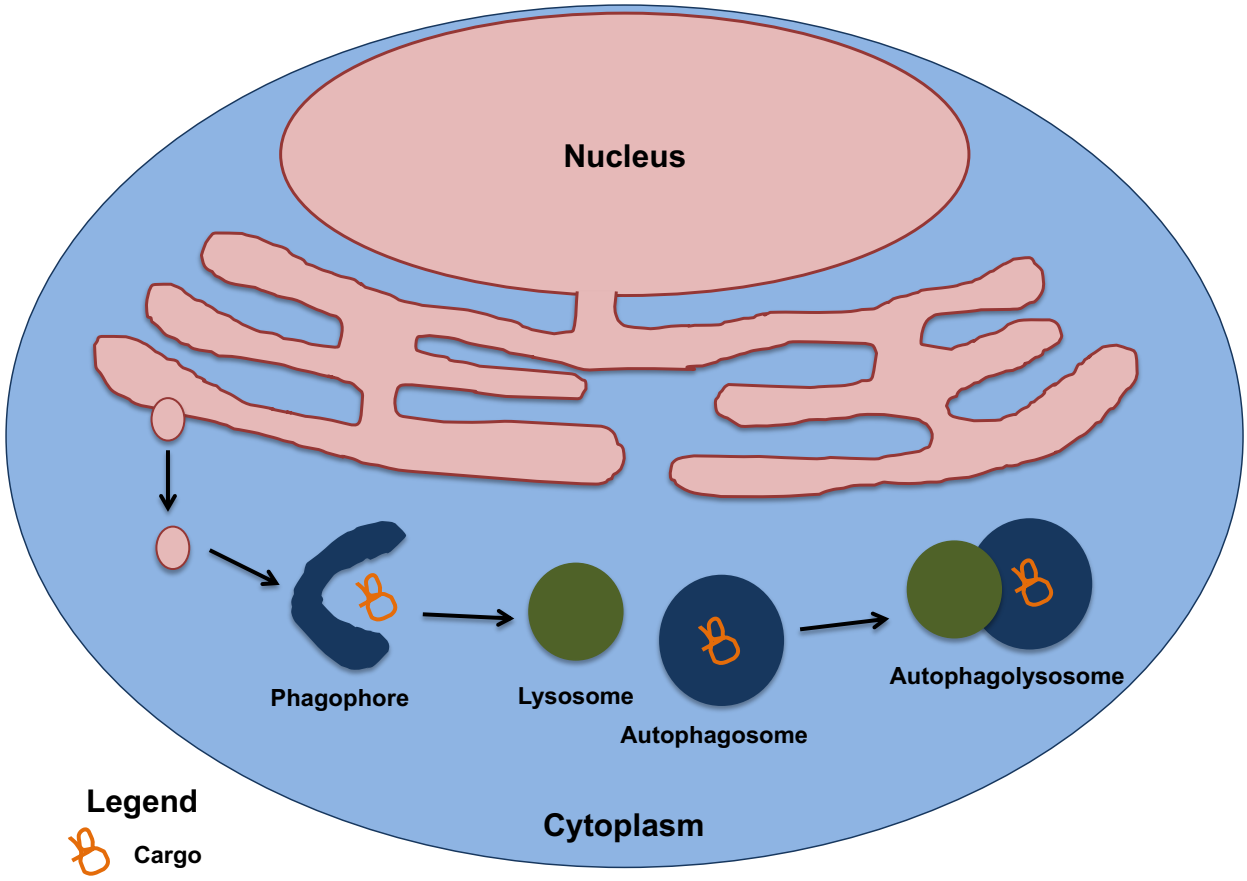


Figure 5: Autophagy model

Autophagy also functions as a survival mechanism, as it can clear damaged organelles and intracellular pathogens (Glick et al. 2010). Moreover, autophagy is known to play a key role in cancer, neurodegenerative diseases, autoimmune diseases, among others, because it can promote cellular senescence, present cell surface antigens, protect against genome instability and DNA damage, and prevent necrosis (Young et al. 2009; English et al. 2009; Karantza-Wadsworth et al. 2007; Degenhardt et al. 2006).

Autophagy is controlled by over 30 autophagy related genes (ATG) that can be activated by lack of nutrients followed by inhibition of the mechanistic target of rapamycin (mTOR) signaling or by the UPR when misfolded proteins accumulate (Mizushima & Komatsu 2011; Deegan et al. 2013). The PERK and IRE1 arms of the UPR have been implicated in activating autophagy (Haberzettl & Hill 2013; B'chir et al. 2013). Moreover, in ATG knockout mice, the IRE1 pathway was active and promotes inflammation, which supports a feedback mechanism between the UPR and autophagy (Deegan et al. 2013; Adolph et al. 2013). In an *in vitro* murine muscle-differentiation system, it was shown that autophagy is induced during skeletal muscle differentiation (Fortini et al. 2016; Levine & Klionsky 2004). Not only is autophagy known to play an important role during bone marrow stem cell differentiation into osteocytes and adipocytes, but it has been shown that autophagy is required for muscle mass maintenance (Masiero et al. 2009; Nuschke et al. 2014). Last, mitophagy, or the selective degradation of mitochondria via autophagy, is required for myogenic differentiation in C2C12 myoblasts (Sin et al. 2016).

Autophagy along with the UPR and circadian clock are known to govern metabolic processes. Disruption of these pathways can cause shifts towards a cancer associated glycolysis and promote cell hyperproliferation (Altman et al. 2015; Fortini et al. 2016; Carracedo et al. 2013). Moreover, it has been shown that autophagy suppresses tumorigenesis in some contexts, but in most contexts, autophagy facilitates tumorigenesis (White 2015; Liang et al. 1999; Takamura et al. 2011; Strohecker et al. 2013; Degenhardt et al. 2006; Guo et al. 2011; Lock et al. 2011). In

some of these cancers, autophagy is upregulated to survive stress, and to promote tumor survival, growth and proliferation (Guo et al. 2011; Lock et al. 2011). Thus autophagy, the UPR and circadian clock potentially offer new therapeutic avenues to treat cancer.

Summary

In summary, the research described in this dissertation will focus on understanding the molecular mechanisms that promote UPS initiation, development, and proliferation. In particular, I sought to elucidate the mechanism by which YAP1 and NF-κB promote sarcomagenesis and disrupt cellular signaling networks important for skeletal muscle differentiation. My findings show the contributions of circadian clock signaling, the UPR, and autophagy have on UPS proliferation and muscle differentiation. For this dissertation, I employed different genetic and epigenetic tools as well as in vitro and in vivo models.

Chapter 2: Materials and Methods

Genetically Engineered Mouse Models (GEMM). All mouse experiments were performed in accordance with NIH guidelines. All protocols and experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. We generated *Kras*$^{G12D+}$; *Trp53*$^{fl/fl}$; *YAP1*$^{fl/fl}$ (KPY) and *Kras*$^{G12D+}$; *Trp53*$^{fl/fl}$; *Rela*$^{fl/fl}$ (KPR) mice by crossing KP with *YAP1*$^{fl/fl}$ and *Rela*$^{fl/fl}$ animals. Tumors were generated by injection of a calcium phosphate precipitate of adenovirus expressing Cre recombinase (University of Iowa) into the right gastrocnemius muscle of 3- to 6-month-old mice.

In vivo drug treatment. For in vivo drug studies, total 44 (n=11 per group) autochthonous KP mice were randomly divided into 4 groups to receive different treatments once tumors reached 100 mm^3 and injected for up to 20 days. The mice are euthanized 24 hrs after the tumor volume reaches 2000 mm^3). 1) Vehicle group (10% Hydroxypropyl-β-cyclodextrin plus DMSO was diluted daily in sterile 45% PEG/55% H_2O); 2). JQ1 and SAHA combination treatment group (drugs were diluted in respective vehicles). Treatment method for drug combination group: 1). 25mg/kg SAHA + 50mg/kg JQ1 for first 5 days. 2) 25mg/kg SAHA+25mg/kg JQ1 each other day for 10days. 3). 25mg/kg SAHA + 50mg/kg JQ1 for 2 days. 4). Then mice with tumors received 25mg/kg SAHA with 25mg/kg JQ1 for 3 days. Mice without tumors received 5mg/kg SAHA and 5mg/kg JQ1 for 3 days). JQ1 was provided by Jun Qi (Dana-Farber Cancer Institute) and SAHA was purchased from Cayman Chemical. HP-β-CD and PEG400 were obtained from Sigma-Aldrich.

Oncomine and TCGA survival analysis. We used the publicly available database Detwiller et al. via the Oncomine Research Premium edition software (version 4.5, life Technologies) to query *PER1, PER2, CRY1, CRY2, ARNTL, TXNIP, DDIT3, FASN, CPT1A, CPT1B* gene expression in MFH/UPS. We also evaluated human patient survival using the TCGA sarcoma dataset. Kaplan-

Meier analyses were performed for overall survival of patients.

Cell Lines. KP230, KP250 and KIA cell lines were derived from UPS mouse tumors as described in {EisingerMathason:2013jn}. Human HT-1080, HEK-293T cell lines were purchased from ATCC (Manassas, VA, USA). STS-109 cell line was derived from human UPS patients. STR analysis was performed at the time of derivation and confirmed in April 2015. Cells were purchased, thawed, and then expanded in the laboratory. Multiple aliquots were frozen down within 10 days of initial resuscitation. For experimental use, aliquots were resuscitated and cultured for up to 20 passages (4–6 weeks) before being discarded. Cells were cultured in DMEM with 10% (vol/vol) FBS, 1% penicillin/streptomycin, 1% glutamine, at 5% CO_2 and 37°C. All cell lines were confirmed to be negative for mycoplasma contamination.

Drug Treatments. Cells were treated with SAHA (2μM) and JQ1 (0.5μM) either individually or in combination for the time indicated in the figure legends. Drugs were refreshed for any cells treated for longer than 48hrs.

Lentiviral Transduction. shRNA-mediated knockdown of Per1: TRCN0000075403; Arntl: TRCN0000095055; Txnip: TRCN0000182360; Ddit3: TRCN0000103709; RelA: TRCN0000055344, TRCN0000055346; and Yap1: TRCN0000095864, TRCN0000095865 were obtained as glycerol stocks from Dharmacon. Scramble shRNA was obtained from Addgene. High-copy plasmid purification was conducted for each shRNA using Clontech Laboratories Inc. NucleoBond Exta Midi kit (740410.50) according to the NucleoBond ® Xtra Plasmid Purification Maxi protocol. shRNA plasmids were packaged by using the third-generation lenti-vector system (VSV-G, p-MDLG, and pRSV-REV) and expressed in HEK-293T cells. Supernatant was collected at 24 and 48 hrs after transfection and subsequently concentrated by using 10-kDa Amicon Ultra-15 centrifugal filter units (Millipore). After 72 hours of lentiviral infection, cells were selected with

puromycin (1.5 μg/mL) for 24-48 hours. shRNA infected cells were treated with SAHA (2μM) and JQ1 (0.5μM) for 48 hours kept under puromycin selecting conditions.

***ChIP-seq*.** For tumor samples resected from UPS patients at the Hospital of the University of Pennsylvania, approximately 100 mg of tissue was minced into 1-2 mm pieces and incubated in 1% formaldehyde for 15 minutes. Formaldehyde was quenched with glycine at 0.125 M. Fixed tissue was homogenized for 60 seconds with a Tissue Tearor Homogenizer (Biospec) at 30,000 RPM. Homogenized tissue was washed with ice-cold PBS with 1X HALT protease inhibitor. For cell-line ChIP-RX, samples were fixed for 10 minutes in 1% formaldehyde quenched with glycine and washed with PBS as above. 5e6 S2 cells (Drosophila Melanogaster) were added to each sample of 2.5e7 for ChIP-RX normalization in downstream analysis.

Transient Transfections. SMARTpool: ON-TARGETplus Yap1 siRNA (M-100439-01-0005), Cry2 siRNA (L-040486-00-0005), Per1 siRNA (L-040487-00-0005), and Non-targeting siRNA were purchased from Dharmacon.

UPR Reporter Assays. KP cells were plated in a 6 well plate and transiently transfected using the Lipofectamine^{TM}3000 protocol. Cells were transfected using the Addgene plasmids ATF4 5: 5'ATF4:GFP (#21852) and pEGFP-ATF6 (#32955). Fluorescence images were taken using Olympus IX2-UCB microscope, SensiCam^{QE} High Perfomance camera, and X-Cite® Series 120PC. Images were taken using the Slidebook 6 program.

Immunoblots. Protein lysate was prepared in SDS/Tris (pH7.6) lysis buffer, separated by electrophoresis in 8-10% SDS/PAGE gels, transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk, and probed with the following antibodies: rabbit anti-PER1 (ab3443; 1:250), rabbit anti-CRY2 (ab38872, 1:500) (Abcam), rabbit anti-BMAL1 (14020S; 1:1000), rabbit anti-YAP1 (4912; 1:1000), rabbit anti-GAPDH (2118; 1:1000), (Cell Signaling Technology), rabbit anti-

CPT1A (15184-1-AP; 1:1000) (Proteintech), rabbi anti-TXNIP (ab188865, 1:1000) (Abcam), rabbit anti-CHOP (60304-1, Ig, 1:1000) (Proteintech), rabbit anti-FASN (3081, 1:1000) (Cell Signaling Technology), anti-LC3A/B (12741, 1:1000) (Cell Signaling Technology), rabbit anti-Caspase-3 (9662; 1:1000).

qRT-PCR. Total RNA was isolated from tissues and cells using the TRIzol regent (Life Technologies) and RNeasy Mini Kit (Qiagen). Reverse transcription of mRNA was performed using the High-Capacity RNA-to-cDNA Kit (Life Technologies). qRT-PCR was performed by using a ViiA7 apparatus. All probes were obtained from TaqMan “best coverage" (Life Technologies). HPRT and or/ SDHA was used as an endogenous control.

Luciferase assay. Plasmid pABpuro-BluF (46824; Addgene) was transfected into 293T cells (ATCC) to generate lentiviral particles in the supernatant. Viral supernatant was harvested and then concentrated by centrifugal filter units (Amicon Ultra-15, Millipore). Then Bmal reporter virus was transduced into KP230 cells. Positive Bmall reporter cells were selected by puromycin. For shRNA assays the Bmal reporter cell line was transduced with lentivirus expressing control or Yap1 shRNA. For drug studies, the Bmal reporter cells were treated with SAHA (2μM)/JQ1 (0.5μM) on time course. Luciferase activity was assayed using the Dual Luciferase Assay System (E2920, Promega) according to the manufacturer’s protocol on a Luminometer (GLOMAX, Promega). Results were calculated as fold induction.

***Microarray and gene set enrichment analysis*.** Microarray services were provided by the UPENN Molecular Profiling Facility, including quality control tests of the total RNA samples by Agilent Bioanalyzer and Nanodrop spectrophotometry. All protocols were conducted as described in the Affymetrix WT Plus Reagent Kit Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 250ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by poly(T) and random oligomers that incorporated the

T7 promoter sequence. Second-strand cDNA synthesis was followed by in vitro transcription with T7 RNA polymerase for linear amplification of each transcript, and the resulting cRNA was converted to cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end labeling. Five and a half micrograms of labeled cDNA were added to Affymetrix hybridization cocktails, heated at 99ºC for 5 min and hybridized for 16 h at 45ºC to Mouse Transcriptome 1.0 ST GeneChips (Affymetrix Inc., Santa Clara CA) using the GeneChip Hybridization oven 645. The microarrays were then washed at low (6X SSPE) and high (100mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A GeneChip 3000 7G scanner was used to collect fluorescence signal. Affymetrix Command Console and Expression Console were used to quantitate expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. Affymetrix cel (probe intensity) files were normalized and summarized using RMA-SST to the gene level using Expression Console software (v1.4.1). Inter sample variation was visualized using Principal Components Analysis in Partek Genomics Suite (v6.6, Partek, Inc., St. Louis, MO). Differential gene expression was tested using Significance Analysis of Microarrays (SAM, samr v2.0), yielding fold change, q-value (false discovery rate) and d-score for each gene. We observed a small number of genes meeting our cutoffs for differential expression and so proceeded to GSEA. Log_2-transformed RMA-sst expression values were used as input to GSEA (Subramanian et al. 2005) where enrichment was tested against the hallmark gene sets from the Molecular Signatures Database (MSigDB, v5.1, http://software.broadinstitute.org/gsea/msigdb/index.jsp)

Accession Codes. Sequencing data reported in this paper have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE109920, and GSE109923.

C2C12 growth and differentiation. C2C12 murine myoblast cells were obtained from ATCC (Manassas, VA, USA). The cells grow as undifferentiated myoblasts in growth medium (20% FBS with 1% penicillin/streptomycin) and were passaged every 2-3 days at 50% sub-confluence. To induce differentiation cells were grown overnight to ~80% confluence in growth medium, and then switched to DMEM supplemented with 2% horse serum. Differentiation media was refreshed every 2 days.

Immunohistochemistry. Human UPS paraffin embedded tissues were obtained from the Surgical Pathology group at Univ. of Pennsylvania and stained TXNIP and CHOP. Murine tumors from the KP GEMM and KP allografts were also sectioned and stained. IHC was performed on 5-micron tissue sections according to standard protocols. Sections were deparaffinized, rehydrated and subjected to epitope retrieval and stained with The following antibody concentrations were used: rabbit anti-TXNIP (ab188865; 1:100) (Abcam), mouse anti-CHOP (Proteintech) (60304-1-IG; 1:250), rabbit anti-LC3B (Novus Biologicals; NB100-2220, 1:1000), rabbit anti-p62 (MBL International, PM045, 1:2000), mouse anti- Gadd34 (Novus Biologicals; NBP2-01787, 1:100) followed by peroxidase-based detection and counterstaining with hematoxylin using the Leica Bond Rx^{m} system with conditions described previously (Berezowska & Galván 2017). Representative photographs were taken on a Leica DMI6000B inverted light and fluorescent microscope with a 40x oil objective. Images were blinded, and positive staining was assessed in Image J. The color deconvolution macro was applied to images resulting in a DAB staining (Color_2) generated window (Ruifrok & Johnston 2001). Using the threshold function, the total area of the tumor in pixels was recorded utilizing the same parameters for each tumor image. Areas staining positive by these parameters were selected and the positive-staining area in pixels was recorded. The positive-staining area of the tumor in pixels was divided by the total area of the tumor in pixels to determine the percentage positive-staining area.

GC/MS. After growth overnight, cultures were treated with either DMSO or 2 μM SAHA/ 0.5 μM JQ1. The cultures were allowed to grow for 48 hours. Subsequently, the medium was changed to DMEM that contained 7 mM glucose, 4 mM glutamine, 75 μM [U-$^{13}C_{16}$]palmitic acid and 75 μM [U-$^{13}C_{18}$]oleic acid plus either DMSO or S/J. Both fatty acids were bound to fatty acid free albumin (2 moles fatty acid per mole of albumin) before they were added to the medium. After 12 hours of growth in the ^{13}C-labeled medium, the cultures were harvested by cold methanol extraction. The cold methanol (80:20 methanol:water) was pre-cooled to -80C and added rapidly (2 ml/dish) after removal of the extraceullar medium to prevent loss of intracellular metabolites. The cultures were stored at -80C prior to gas chromatography/mass spectrometry analysis (GC/MS) analysis. The methanol/cell mixtures were sonicated for 60 seconds with a probe sonicator to disrupt all cell membranes and then centrifuged at 13,000xg for 10 minutes. The supernatants were removed and transferred to sealable 4-ml glass tubes. Methanol and water were removed from the cell extracts with a heated (45C) nitrogen evaporator. For GC/MS, the extracts were first derivatized with N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA). The extracts in the 4-ml glass tubes were dissolved in 60 μL of pyridine. Subsequently, 60 μL BSTFA with 1% trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO) were added and the mixtures were heated to 55C for 60 min. After cooling, the reaction mixtures were centrifuged at 13,000xg for 5 minutes. The supernatant was transferred to 1.5 ml capped injection vials that were fitted with volume reducing glass sleeves. The derivatized samples were analyzed with an Agilent 7890A/5975C GC/MS system. Mass fragments were generated by electron impact at 70eV. Helium was used as the carrier gas for the GC DB-5 column (30 m, with 10 m empty pre-column) at a flow rate of 1 ml/min. The injector was operated in splitless mode at 250C. The column temperature profile was 0-3 min: 100C, 3-17 min: ramp 10C/min, 17-47 min: 240C, 47-52min: 300C. The citrate retention time was 24.1 minutes. The mass scan range was 50 to 550 daltons. The relative enrichment of metabolites was calculated using IsoCor (Millard et al. 2012).

Statistical Analysis. Statistical analysis was performed using Prism (Graph Pad Software). Data are shown as mean ± SEM or SD. Data were reported as biological replicates. Experiments were performed in triplicate. Student *t*-tests (unpaired two tailed) were performed to determine whether a difference between two values is statistically significantly different, with a *P*-value <0.05 considered significant.

Chapter 3: YAP1 enhances NF-κB-dependent and independent effects on clock-mediated unfolded protein response and autophagy in sarcoma

Introduction

Soft-tissue sarcomas (STS) are a complex set of tumors that arise in mesenchymal tissues, including muscle, fat, cartilage and connective tissue. The roughly 65 known sarcoma subtypes can be generally divided into two groups 1) majority pediatric sarcomas which most frequently develop as a result of chromosomal translocations (Kadoch & Crabtree 2013; Ciarapica et al. 2014; Shern et al. 2014) and 2) typically adult sarcomas that have no known consistent oncogenic mutations, but ~50% of which bear mutations in tumor suppressor genes like RB1 or p53 (W. Lee et al. 2014; De Raedt et al. 2014; M. Zhang et al. 2014). Due to their karyotype complexity, variety of subtypes, and the lack of known drivers, adult sarcomas are very poorly understood. Treatment options are generally limited to radiation and surgery, as inadequate characterization has precluded the development of targeted therapies (Pappo et al. 2014; Wagner et al. 2015; Taylor et al. 2011). Our current work focuses on undifferentiated pleomorphic sarcoma (UPS), an aggressive adult tumor found in skeletal muscle. Muscle-derived UPS is a commonly diagnosed subtype relative to other sarcomas and is particularly difficult to treat (Ballinger et al. 2016). Patients rely on radiation and resection, which can be traumatic and frequently fails to prevent metastasis. My work shows that the central Hippo effector, Yes associated protein 1 (YAP1), is stabilized in human UPS tumors and promotes a pro-proliferation transcriptional program (Mizuno et al. 2012; Ye et al. 2018). YAP1 is unusually stable in UPS and potentially other sarcomas due to epigenetic silencing of its inhibitor, Angiomotin (AMOT), and Hippo kinase copy number loss (Rubin et al. 2011; Eisinger-Mathason et al. 2015). These perturbations stabilize YAP1 at the protein level; enhance its nuclear localization and subsequent transcriptional activity (Ye et al. 2018).

Though well studied in epithelial tumors, the specific downstream effectors of YAP1 in sarcomas are not well characterized. Skeletal muscle-derived UPS is thought to develop from muscle progenitor cells/satellite cells, which undergo proliferation as immature myoblasts before differentiating into mature muscle fibers. YAP1 and NF-κB signaling are essential for myoblast proliferation and these pathways must be inhibited to permit terminal differentiation (Rubin et al. 2011; Watt et al. 2010; Tubaro et al. 2010; Bakkar et al. 2008; Dogra et al. 2006; Ardite et al. 2004). Thus, during normal muscle development inhibition of NF-κB and YAP1 are associated with loss of proliferative capacity, and upregulation of muscle differentiation markers like MYOD and MEF2C. Recently we discovered that YAP1 controls NF-κB activity in muscle-derived UPS, by inhibiting expression of Ubiquitin Specific Peptidase 31 (USP31) a negative regulator of NF-κB (Ye et al. 2018). In the absence of a specific inhibitor for YAP1 we used a combination of the epigenetic modulators suberoylanilide hyroxamic acid (SAHA; Vorinostat), and the BET bromodomain inhibitor JQ1, which we recently discovered effectively suppresses YAP1 activity by upregulating the YAP1 cytoplasmic sequestration protein AMOT and reducing YAP1 expression. Though SAHA/JQ1 treatment has widespread effects, we use these tools to interrogate and then validate YAP1-mediated signaling and phenotypes. Importantly, SAHA/JQ1 treatment upregulated a transcriptional program associated with muscle differentiation in these tumors. Consistently, here we report that shRNA-mediated inhibition of YAP1 and/or NF-κB recapitulates several key aspects of SAHA/JQ1-mediated differentiation.

Interestingly, we observed that (Zambrano et al. 2016; X. Wang et al. 2015). Consistent with these findings, normal myoblast proliferation and muscle differentiation have been linked to peripheral circadian oscillation (Andrews et al. 2010; X. Zhang et al. 2009; McCarthy et al. 2007). The circadian clock is a 24-hour molecular signaling hub that regulates proliferation via control of metabolic processes and is regulated by positive and negative feedback loops (Bass & Takahashi 2010; Huang et al. 2016; Bell-Pedersen et al. 2005; Lowrey & Takahashi 2004). The main

transcriptional components, CLOCK and BMAL1, form a heterodimer that binds to an E-box in the promoters of target genes, such as *PERIOD* (*PER*) and CRYPTOCHROME (*CRY*), promoting their expression, which in turn inhibit CLOCK and BMAL1 heterodimers (Lowrey & Takahashi 2004; Ripperger & Schibler 2006). Shuttling of PER and CRY proteins from the cytoplasm to the nucleus regulates the feedback loop. Importantly, a growing body of literature suggests that disruption of circadian oscillation promotes tumorigenesis in a variety of cancer settings (S. Wu et al. 2016; Ha et al. 2016; Fu et al. 2002).

Recent studies have shown that clock activity is linked to a number of pathways associated with muscle differentiation including the Unfolded Protein Response (UPR) (Bu et al. 2018; Igarashi et al. 2007; Huang et al. 2016; Fortini et al. 2016; W.-S. Lee et al. 2015; Masiero et al. 2009). Crosstalk between (Margariti et al. 2013; Gade et al. 2012; Rzymski et al. 2010) . Importantly, YAP1 plays a role in cancer related autophagic flux a(Q. Song et al. 2015; H. Wu et al. 2015). Based on these observations we hypothesized that YAP1 and/or NF-κB helps control differentiation in muscle tissues and tumors via regulation of the circadian clock and downstream processes. My work demonstrates that the YAP1/NF-κB axis controls the switch between differentiation and proliferation in sarcoma by suppressing the circadian clock and UPR. Interestingly, I observed that YAP1 also suppresses autophagy but in an NF-κB-independent manner.

Results

Clock gene expression is high in differentiating muscle and lost in UPS

We investigated the role of YAP1 in UPS, by silencing it in our genetically engineered murine model (GEMM) of UPS. We introduced *Yap1*$^{fl/fl}$ alleles into the *LSL-Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$ (KP) system to generate *LSL-Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$; *Yap1*$^{fl/fl}$ (KPY) animals. The KP model generates sarcomas that recapitulate human UPS and is thus the standard GEMM used in these studies (Kirsch et al. 2007; Mito et al. 2009). Importantly, Yap1 protein expression is stabilized in KP tumors5, which provides further rationale for the study of Yap1 in sarcoma using this model (Ye et al. 2018). Injection of adenovirus expressing Cre recombinase into the right gastrocnemius muscle initiates tumorigenesis. The Cre recombinase activates expression of oncogenic Kras while simultaneously deleting p53 and/or Yap1 in infected muscle progenitor cells (Kirsch et al. 2007; Mito et al. 2009). Though *Kras* mutation is rare in human sarcomas, *Trp53* mutation and deletion are very common (Cancer Genome Atlas Research Network. Electronic address: elizabeth.demicco@sinaihealthsystem.caCancer Genome Atlas Research Network 2017). Additionally, hyperactivation of the MAPK pathway, downstream of KRAS activation, is also common in UPS and is an excellent prognostic indicator for recurrence (Serrano et al. 2016). Yap1 loss in KPY tumors increases latency and reduces tumor weight and volume (Ye et al. 2018). To determine the functional role of Yap1 in UPS we performed microarray analysis of 5 individual KP and KPY tumors. In addition to suppression of NF-κB targets (Ye et al. 2018), we observed differential expression of the circadian clock genes *Per1, Per2, Cry1, Cry2* and *Arntl* (**Figure 6a**), validated by qRT-PCR of RNA isolated from the murine tumors (**Figure 6b**). Because circadian clock function is important for skeletal muscle mass maintenance (Kondratov et al. 2006) and CLOCK and BMAL1 regulate MYOD, the master regulator of muscle differentiation (Andrews et al. 2010), we next queried clock gene expression in differentiating myoblasts using the C2C12 system. In C2C12 murine myoblasts, differentiation media containing 2% horse serum activates myogenesis (**Figure 6c**). We observed an increase in expression of the Yap1 inhibitor *Amot*, clock genes (*Per1, Per2*) and the NF-κB negative regulator, Usp31 in

differentiated C2C12 cells (**Figure 6d**). Per1 and Per2 protein expression corresponded to loss of Yap1 in differentiating C2C12 cells (**Figure 6e**), indicating that the circadian clock is upregulated during muscle differentiation. Using Oncomine we queried publicly available sarcoma datasets for expression of *PER1, PER2*, and *CRY2* in fibrosarcoma (FS) and UPS. Some fibrosarcomas, including myxofibrosarcomas (MFS), are now thought to be genetically indistinguishable from UPS (Cancer Genome Atlas Research Network. Electronic address: elizabeth.demicco@sinaihealthsystem.caCancer Genome Atlas Research Network 2017) and are therefore included in our studies. We found that clock genes are suppressed in these tumors compared to skeletal muscle and other normal tissues (**Figure 6f-h**). Together, these data suggest that Yap1 represses clock gene expression in muscle-derived UPS and that circadian clock is important for muscle differentiation. Based on these observations we hypothesized that expression of one or more circadian genes is linked to survival of patients with UPS/MFS. Based on these observations we hypothesized that expression of one or more circadian genes is linked to survival of patients with UPS/MFS.

We investigated the relationship between long-term survival and expression of *PER1* or *CRY2*, which are most consistently upregulated by YAP1/NF-κB loss in our studies. Importantly, our analysis of human MFS samples from The Cancer Genome Atlas (TCGA) showed that low levels of *CRY2* are associated with poor survival (**Figure 7a**). To assess *CRY2* expression in these tumors, relative to normal muscle, we performed RNA-seq and ChIP-seq of human skeletal muscle and multiple human UPS samples. *CRY2* expression is suppressed in UPS tumors compared to muscle (Ye et al. 2018). Consistent with this finding we found that enrichment of Histone 3-lysine 27 Acetylation (H3K27Ac) is lost in UPS relative to skeletal muscle at the *CRY2* locus (**Figure 7b**). H3K27Ac enrichment is associated with active transcription and potential enhancer activity. The *PER1* locus shows a similar pattern of H3K27Ac enrichment/gene expression in muscle and loss in UPS (**Figure 7c)** but there was no statistically significant link

between *PER1* and patient survival, suggesting the relatively low number samples may be affecting our analysis in some cases.

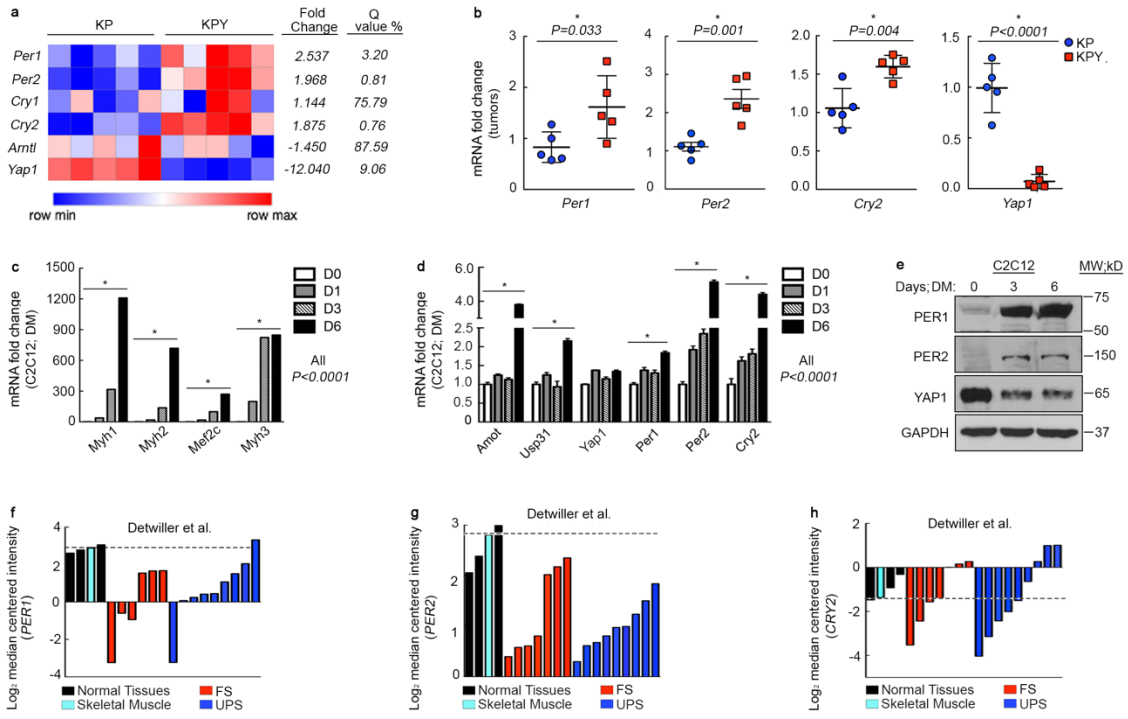


Figure 6: Yap1-dependent inhibition of circadian clock genes in UPS and proliferating myoblasts

A) Gene expression analysis of microarray performed on KP vs. KPY mouse tumors.
B) qRT-PCR validation of circadian clock gene expression in KP and KPY mouse tumors.
C) qRT-PCR of muscle differentiation genes.
D) qRT-PCR of Hippo/NF-κB/Circadian clock genes in proliferating (Day 0, D0) and differentiating (D1-D6) C2C12 myoblasts.
E) Western blot of Per1 and Per2 in C2C12 cells treated as in D.
F) Oncomine gene expression analysis of *PER1* in human tissues.
G) Oncomine gene expression analysis of *PER2* in human tissues.
H) Oncomine gene expression analysis of *CRY2* in human tissues.

*Shaui Ye, Gloria Marino, Shaun Egolf, Gabrielle E. Ciotti, Ying Liu, and Susan Chor helped by performing experiments and analyzing data.

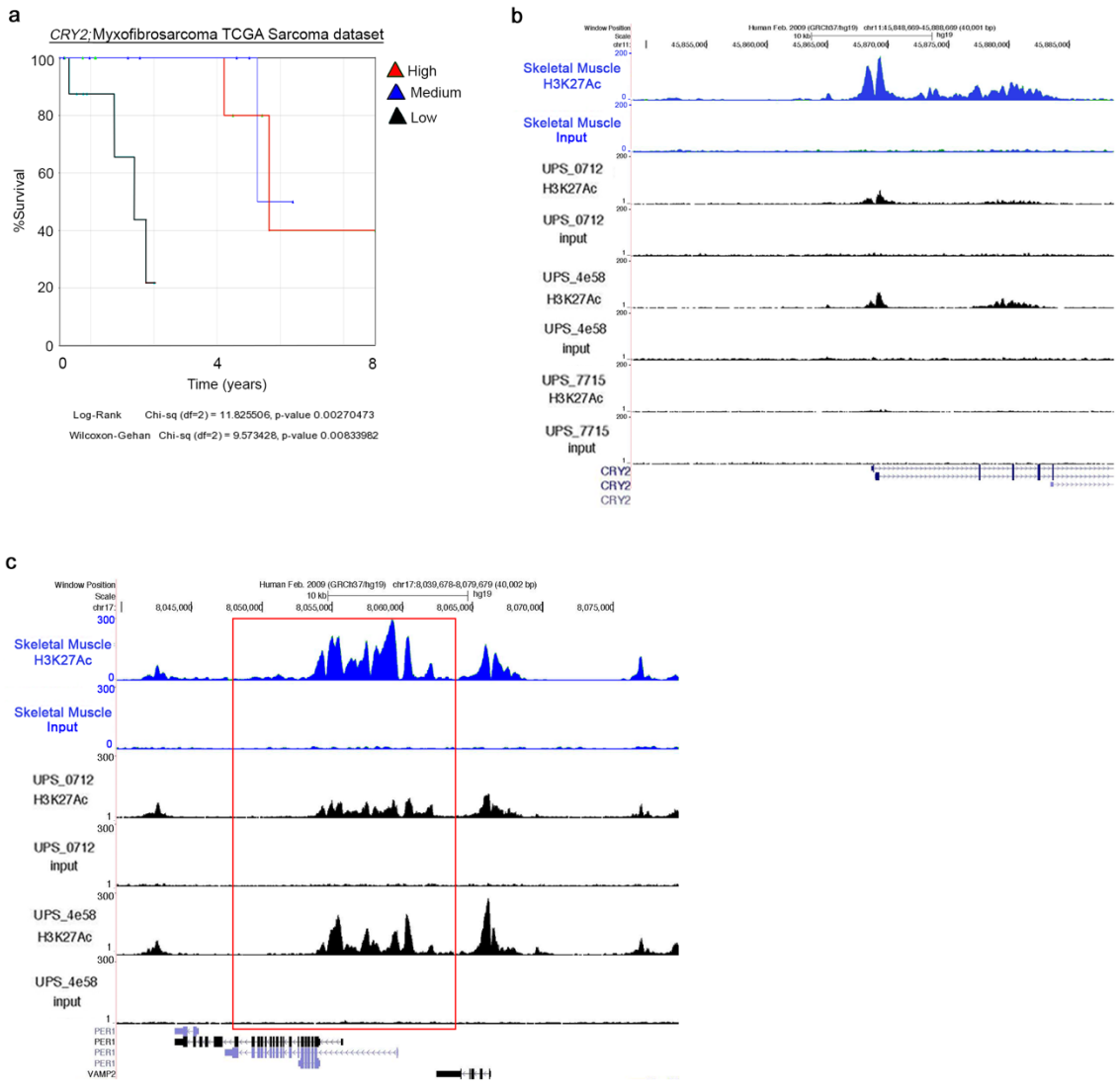


Figure 7: Clock genes in human UPS tumors

A) Kaplan Meier survival curve of MFS patients in the TCGA sarcoma dataset based on *CRY2* expression.
B) Gene tracks of H3K27ac ChIP-seq signal (rpm/bp) for H3K27ac at the *CRY2* locus in human skeletal muscle and human 3 independent human UPS samples. Error bars represent SD.
C) Gene tracks of H3K27ac ChIP-seq signal (rpm/bp) for H3K27ac at the *PER1* locus in human skeletal muscle and human 3 independent human UPS samples.

*Shuai Ye and T.S. Karin Eisinger helped by performing experiments and analyzing data. John Tobias assisted in the data analysis. Human tumor samples were provided by Kristy Weber.

YAP1 suppresses circadian clock gene expression in UPS

We previously reported that YAP1 expression and transcriptional activity is downregulated in sarcoma cells treated with SAHA/JQ1 *in vitro* and *in vivo* and that ectopic expression of constitutively nuclear YAP1 (YAPS6A mutant) can rescue ~40 of the proliferation defects associated with epigenetic modulation (Ye et al. 2018). Based on these observations, we hypothesized that SAHA/JQ1 treatment would reactivate clock gene expression due to loss of YAP1. Thus, we performed microarray analysis and compared gene expression in untreated vs. SAHA/JQ1 treated KP cells derived from KP tumors. We observed differential expression of circadian clock genes (**Figure 8a**). We further validated *Per1, Cry2*, and *Arntl* (*Arntl* encodes the protein Bmal) upregulation via qRT-PCR (**Figure 8b**) and Western blot (**Figure 8c)** of KP and human UPS cells (STS-109) (**Figure 8d and e)**. *PER1* mRNA levels also increased in SAHA/JQ1 treated HT-1080 human fibrosarcoma cells (**Figure 8f**). The subset of clock genes regulated by YAP-deletion (KPY) *in vivo* and SAHA/JQ1-treatment *in vitro* varied slightly. We hypothesized that this target variation was due to the difference in approaches we expressed YAP1-specific shRNAs in KP cells and evaluated clock targets. We found that YAP1 inhibition phenocopied SAHA/JQ1 treatment *in vitro* (**Figure 8g**). We went on to validated PER1 and CRY2 antibodies, using siRNA to determine the appropriate Western blot bands (**Figure 8h and i**).

To determine if YAP1 inhibition functionally induces clock activity we performed a Bmal luciferase reporter assay in KP cells using *Yap1*-specific siRNA, shRNA and SAHA/JQ1 (**Figure 9a-c**). We observed increased Bmal reporter expression in all three systems, indicating that Bmal/circadian clock activity is under the control of YAP1. To verify that clock activity is responsible for inhibition of proliferation in Yap1-depleted cells we performed an *in vitro* rescue assay using a combination of *Yap1*-specific and *Arntl*-specific shRNAs. We observed a ~40% proliferation rescue in double knockdown cells relative to cells expressing YAP1 shRNA alone (black bar vs. gray bar) (**Figure 9d, left**), which correlated with a ~40% reduction of *Arntl* in double knockdown cells relative to cells expressing YAP1 shRNA alone (**Figure 9d, right and**

9e) (black bar vs. gray bar). Previously, we reported that Yap1-depletion promotes muscle-like differentiation (Ye et al. 2018). Here we report that Yap1 loss enhances cell cycle arrest in KP cells (**Figure 9f)**. The number of KP cells in S phase drops 20-45% in cells expressing Yap1 shRNAs. We conclude that the percentage of cells arrested outside of S-phase is roughly equivalent to the percentage of cells rescued by inactivation of the clock. Yap1 is also known to inhibit apoptosis via upregulation of Survivin/*Birc5.* Consistently, we have observed that SAHA/JQ1 treatment abolishes *Birc5* expression. Therefore, we interrogated the role of Yap1 in apoptosis in KP cells. We found that shRNA-mediated inhibition of Yap1 expression increased cleavage of caspase 3, indicating enhanced apoptosis (**Figure 9g**). Importantly, apoptotic cells are unlikely to re-enter the cell cycle in response to *Arntl* suppression (**Figure 9d and e**). Thus, while our rescue phenotype may appear modest, we conclude that we are rescuing a significant fraction of non-apoptotic cells. Together, these data suggest that Yap1-mediated repression of clock gene expression promotes cell cycle progression and suppresses apoptosis, leading to an overall increase in cell numbers. Inhibition of clock function can partially reverse this phenotype, highlighting the importance of circadian function is UPS proliferation.

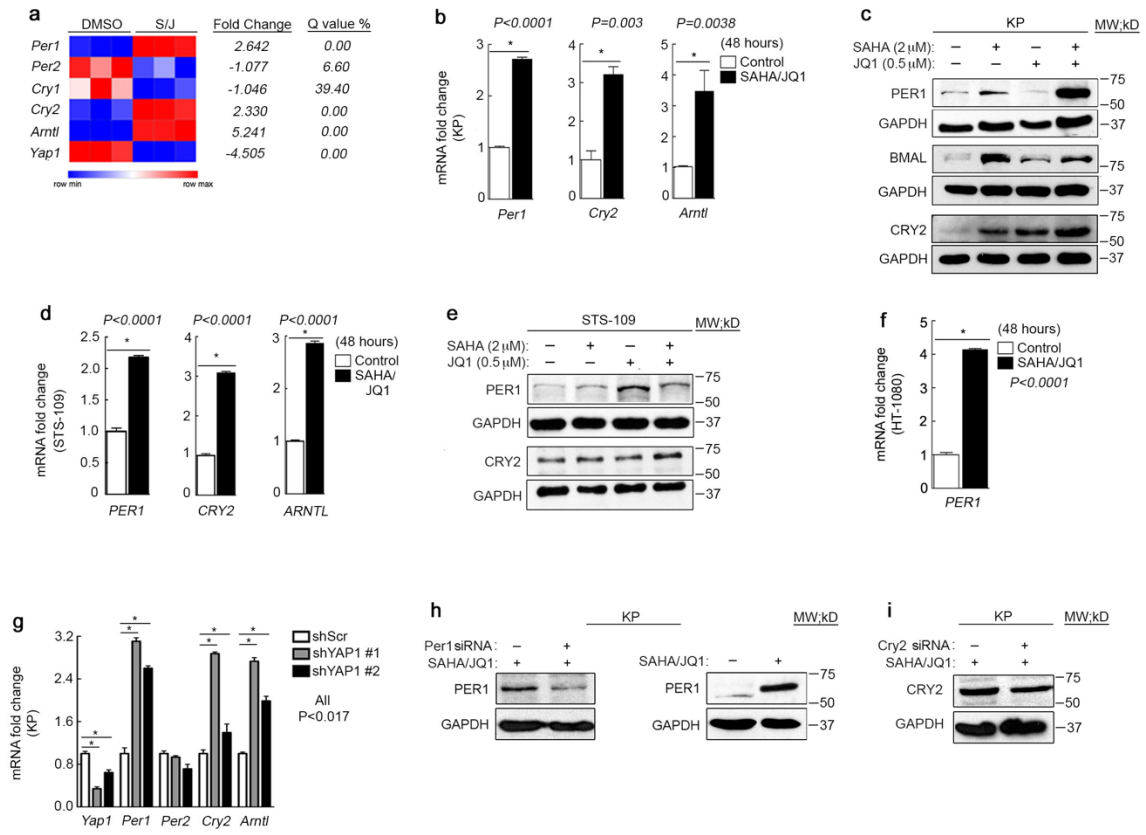


Figure 8: Yap suppresses clock gene expression

A) Gene expression analysis of microarray performed on KP cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
B) qRT-PCR validation of clock genes in KP cells treated as in A.
C) Western blot of KP cells treated as in A.
D) qRT-PCR of human UPS cells (STS-109) treated as in A.
E) Western blot of STS-109 cells treated as in A.
F) qRT-PCR of HT-1080 cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
G) qRT-PCR of KP cells expressing YAP1 shRNA.
H) Western blot of KP cells treated as in B with or without PER1 siRNA to identify the correct band.
I) Western blot of KP cells treated as in B with or without Cry2 siRNA to identify the correct band.

*Shaui Ye, Gloria Marino, Shaun Egolf, Gabrielle E. Ciotti, and Susan Chor helped by performing experiments and analyzing data.

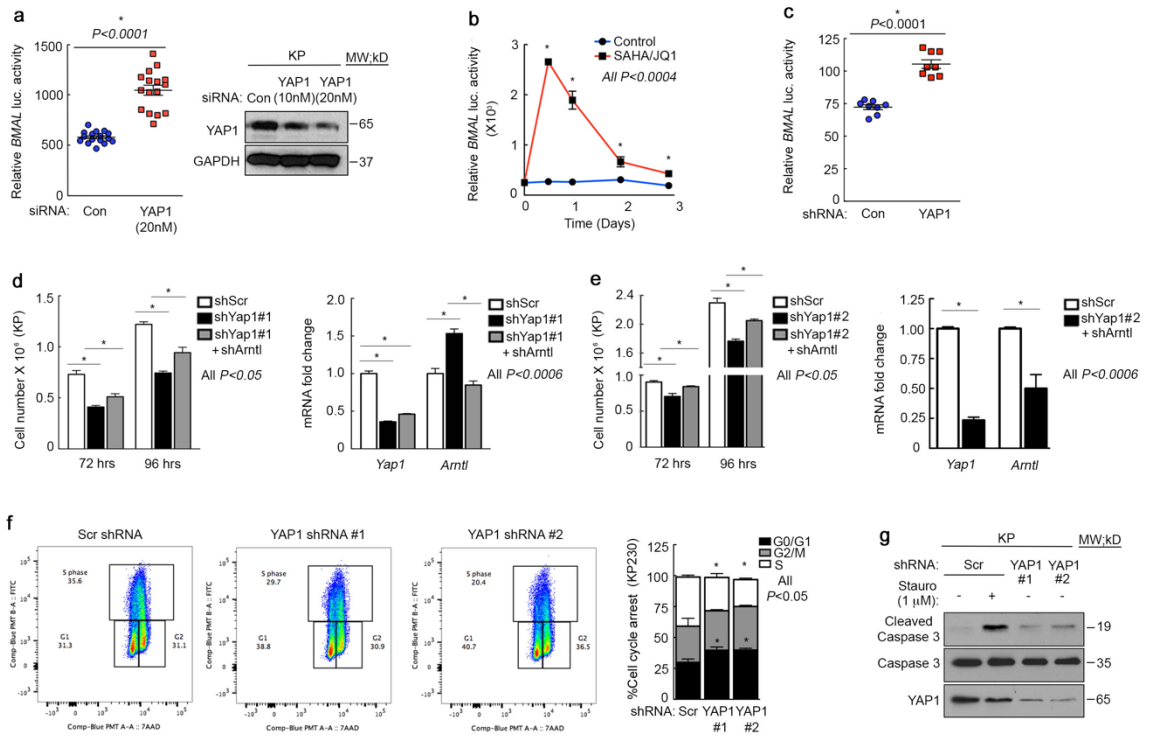


Figure 9: Yap-mediated suppression of the clock enhances sarcoma proliferation

A) (left) Bmal luciferase reporter assay in KP cells expressing *Yap1* siRNA (20nM) and treated with 2 μM SAHA/ 0.5 μM JQ1 for 12 hrs to activate signaling. (right) Western blot of YAP1 levels in siRNA treated cells.

B) Bmal luciferase reporter assay in KP cells treated with 2 μM SAHA/ 0.5 μM JQ1.

C) Bmal luciferase reporter assay in KP cells expressing *Yap1* shRNA.

D) (left) Cell counting rescue proliferation assay in *Yap1#1* and *Arntl* shRNA expressing KP cells. (right) qRT-PCR of *Yap1* and *Arntl* expression in KP.

E) (left) Cell counting rescue proliferation assay in *Yap1#2* and *Arntl* shRNA expressing KP cells. (right) qRT-PCR of *Yap1* and *Arntl* expression in KP cells treated as in D.

F) Representative flow cytometry plots and quantitation of BrdU incorporation in HT-1080 cells expressing multiple independent YAP1 shRNAs.

G) Western blot of KP cells expressing multiple YAP1 shRNAs and treated with 1 μM Staurosporin (12 hr) as positive control. Error bars represent SD.

*Shaui Ye, Gabrielle E. Ciotti, and Susan Chor helped by performing experiments and analyzing data.

Circadian clock genes are regulated by NF-κB downstream of YAP1

We previously reported that YAP1 enhances proliferation in part via upregulation of persistent NF-κB signaling (Ye et al. 2018). Here we interrogated the role of NF-κB in UPS initiation using the KP system. We bred *Rela*$^{fl/fl}$ mice into our KP model and found that genetic deletion of NF-κB, encoded by the *Rela* gene prevents outgrowth of tumors (**Figure 10a and b**). These findings are in agreement with our earlier observation that expression of p65 specific shRNAs reduces KP tumorigenesis in an *in vivo* allograft model (Ye et al. 2018). NF-κB activity is directly linked to p65 phosphorylation because p65 is a critical subunit of the NF-κB transcription factor. Phosphorylated p65 translocates to the nucleus and promotes transcription. Both KP tumors and subcutaneous xenografts of human UPS cells stain positively for YAP1 and p-p65, supporting the hypothesis that this signaling axis is critical for growth in human and murine UPS and can be modeled with these tools (**Figure 10c**). Using pharmacological and genetic approaches we investigated whether YAP1 repression of clock genes is mediated by NF-κB activity. NF-κB inhibition with BAY 11-7085 (1 μM) for 48 hours increased *Per1, Cry2,* and *Arntl* mRNA expression (**Figure 10d**). shRNA-mediated Rela inhibition recapitulates this finding (**Figure 10e**). Importantly, NF-κB target expression oscillates over time in differentiating myoblasts (Ye et al. 2018) and other cells (Zambrano et al. 2016). YAP1 suppresses NF-κB oscillation by controlling expression of USP31 (Ye et al. 2018). Based on these findings, and our observation that *Usp31* also oscillates in differentiating C2C12 cells (**Figure 10d**), we hypothesized that *USP31* might oscillate on a 24-hour clock upstream of circadian clock gene expression. We treated human STS-109 (**Figure 10f**), KP, and HT-1080 cells (**Figure 10g**) with SAHA/JQ1 for 0-120 hours and found that treatment restores oscillation of *USP31* in all three UPS cell lines. Interestingly the period of oscillation varies with the proliferation rate of the cells. Slower proliferation rates (KP>HT-1080>STS-109) correlate with shorter time to maximal USP31 induction by SAHA/JQ1. Ultimately *Usp31* levels are lost in KPY tumors relative to KP (**Figure 10h and i**). This finding is consistent with our Bmal luciferase reporter assay in which we observe

that SAHA/JQ1 treatment initially induces clock activity, which then returns to low levels (**Figure 9a**). Our findings suggest the provocative hypothesis that NF-κB oscillation may drive the circadian cycle in muscle and muscle-derived sarcoma subtypes. To verify that clock activity is responsible for loss of proliferation in NF-κB-depleted cells we performed an *in vitro* rescue assay using a combination of *Rela*-specific and *Arntl*-specific shRNAs (**Figure 10j and k**). We observed a ~20% increase in proliferation (black bar vs. gray bar) (**Figure 10j, left**), which correlates with a ~40% reduction of *Arntl* in double knockdown cells (black bar vs. gray bar) (**Figure 10j, right**). Similarly, we applied BAY 11-7085 (1 μM) for 72 hours to KP cells expressing Arntl specific shRNA. BAY treatment dramatically decreased cell proliferation. Importantly, Arntl inhibition rescued a significant amount of proliferation in BAY treated KP cells (**Figure 10k**). These data indicate that *Rela*-mediated suppression of the clock enhances proliferation, a process that can be partially reversed by inhibiting clock function. We conclude that NF-κB activity controls tumorigenesis in this system, which may be due in part to regulation of the circadian clock.

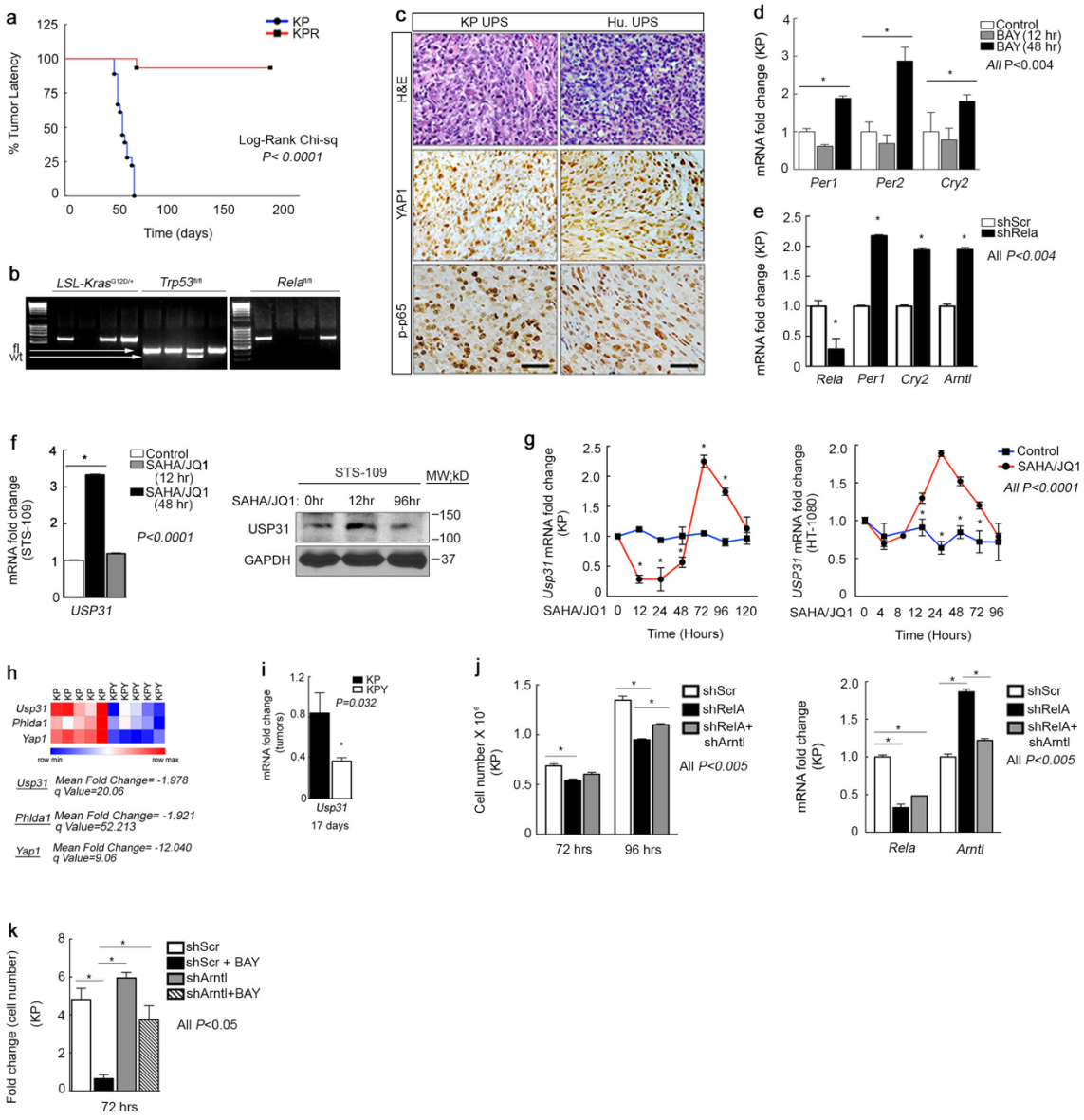


Figure 10: Inhibition of NF-κB, downstream of Yap1, restores clock gene expression

A) *Rela* deletion in the KP autochthonous model of UPS (n=18 mice per group. Log-Rank Chi-sq).
B) Genotyping of KP and KPR mice. Kras band indicates the presence of the *Kras*^{G12D} mutant allele, p53 bands indicate wt and fl/fl alleles. RelA band indicates the presence of the fl/fl alleles.
C) IHC of KP and human xenograft UPS tumors.
D) qRT-PCR of KP cells treated with NF-κB inhibitor 1.5 μM BAY 11-7085 for 12, 48 hrs.
E) qRT-PCR of clock genes in KP cells expressing Rela shRNA.
F) (left) qRT-PCR and (right) Western blot of *USP31* expression in 2 μM SAHA/ 0.5 μM JQ1 treated STS-109.

G) qRT-PCR of KP and HT-1080 cells treated with DMSO or 2 μM SAHA/ 0.5 μM JQ1 for 0-120 hrs.
H) Gene expression analysis of microarray performed on KP vs. KPY mouse tumors. The NF-κB target, *Phlda1*, is included as a control.
I) qRT-PCR validation of *Usp31* gene expression in KP and KPY mouse tumors.
J) (left) Cell counting rescue proliferation assay in *Rela* and *Arntl* shRNA expressing KP cells. (right) qRT-PCR of *Rela* and *Arntl* expression in KP cells treated as in left.
K) Cell counting rescue proliferation assay in *Arntl* shRNA expressing KP cells treated with 1.0 μM BAY 11-7085 for 72 hr. Data is expressed as mean fold change and SEM relative to the cell numbers in samples treated for with DMSO or BAY for 24 hrs. Error bars represent SD.

*Shaui Ye, Gabrielle E. Ciotti, and Susan Chor helped by performing experiments and analyzing data. Human tumor samples were provided by Kristy Weber.

YAP1/NF-κB loss initiates a muscle differentiation-associated UPR response

Recent studies have established a link between the UPR and the clock (Bu et al. 2018; Yuan et al. 2017). Based on these observations and our finding that SAHA/JQ1 treatment activates clock genes (**Figure 8a**) we evaluated UPR activity and found that SAHA/JQ1 treatment also upregulates multiple UPR target genes based on microarray analyses (**Figure 11a**). Transcription of multiple PERK and ATF6- associated UPR targets were upregulated. We validated induction of PERK and ATF6 pathways; two of three UPR branches, in SAHA/JQ1 treated cells (left) and cells expressing multiple independent Yap1 shRNAs (right) via qRT-PCR (**Figure 11b)**. The third branch is IRE1-dependent. However, we did not observe mRNA upregulation or splicing in the IRE1 pathway specific target, *Xbp1* (**Figure 11c**). *Txnip* and *Ddit3*, which encodes the UPR target Chop, are well studied PERK and ATF6 transcriptional targets. Importantly, these targets are two of the most differentially expressed genes from the DMSO vs. SAHA/JQ1 microarray. Using Oncomine, we observed that *Txnip* and *Ddit3* expression are elevated in skeletal muscle and other tissues, but are suppressed in fibrosarcomas and UPS, suggesting that the UPR antagonizes sarcomagenesis in muscle-derived tumors (**Figure 11d and e**). Consistent with these observations, TXNIP and CHOP protein expression are elevated in SAHA/JQ1 treated KP and HT-1080 cells (**Figure 11f-i**). We sought to determine if *Txnip* and *Ddit3* transcription are regulated specifically by YAP1. We inhibited *Yap1* with specific shRNAs and observed increased *Txnip* and *Ddit3* expression (**Figure 11i**).

To confirm upregulation of UPR targets in muscle specifically, we evaluated *Txnip* and *Ddit3* in our C2C12 model of myoblast differentiation and observed increased expression in differentiated cells (**Figure 12a**). To determine if NF-κB mediates *Txnip* and *Ddit3* expression downstream of Yap1 we treated KP cells with BAY 11-7085 or expressed *Rela* shRNAs and observed an increase in *Txnip* and *Ddit3* mRNA expression (**Figure 12b and c**). Next, we performed a rescue experiment to determine if YAP1-mediated suppression of the circadian clock controls expression of Txnip and Ddit3. We treated KP cells with SAHA/JQ1 and *Per1* shRNA

alone or in combination. Interestingly, *Txnip* induction is similarly dependent on clock activity, whereas *Ddit3* expression is not (**Figure 12d**) indicating that *Ddit3* regulation may have clock-independent aspects. Importantly, this observation agrees with our evaluation of *Txnip* and *Ddit3* levels in differentiating C2C12 cells, wherein *Txnip* oscillates during a 6-day differentiation time course but *Ddit3*, simply increases at each time point (**Figure 12a**). To ascertain the functional role of key UPR targets in SAHA/JQ1-induced differentiation we silenced *Txnip* and *Ddit3* with specific shRNAs. We observed that loss of *Txnip* increases the expression of *Ddit3* and the converse is true as well (**Figure 12e**). Therefore, we silenced both *Txnip* and *Ddit3* in SAHA/JQ1 treated cells to prevent compensation. Loss of *Txnip* and *Ddit3* in SAHA/JQ-treated cells alters the normally cytostatic effects of these inhibitors and results in cell death, suggesting that UPR activation supports cell survival in differentiating muscle and sarcoma cells (**Figure 12f and g**). Thus, we conclude that the Yap1/NF-κB axis represses activation of targets in the PERK and ATF6 arms of the UPR, which are associated with clock activity and survival during differentiation (Bu et al. 2018; Yuan et al. 2017; Bohnert et al. 2018; Xiong et al. 2017).

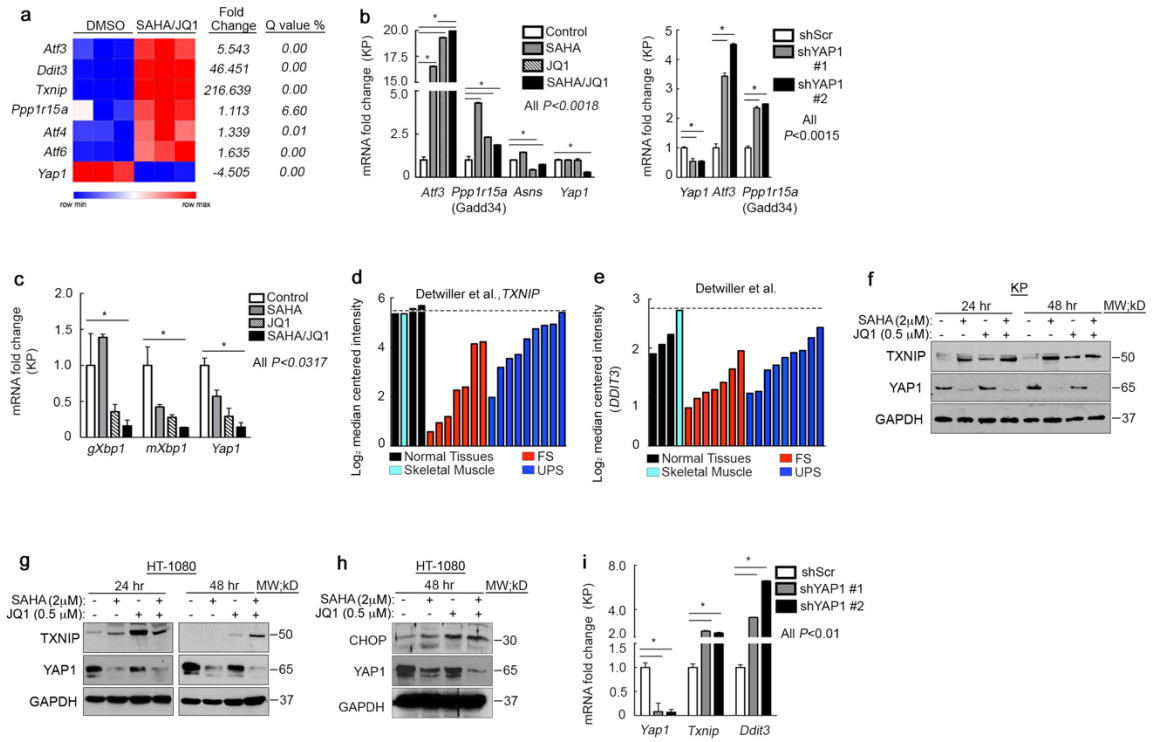


Figure 11: Inhibition of YAP1 and NF-κB activates UPR target gene expression

A) Gene expression analysis of microarray performed on KP cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
B) qRT-PCR validation of UPR genes in (left) KP cells treated as in A and (right) with multiple independent YAP1 shRNAs.
C) qRT-PCR of KP cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
D) Oncomine gene expression analysis of *TXNIP* in human tissues.
E) Oncomine gene expression analysis of *DDIT3* in human tissues.
F) Western blot of TXNIP in KP cells treated as in A.
G) Western blot of TXNIP in HT-1080 cells treated as in A.
H) Western blot of CHOP in HT-1080 cells treated as in A.
I) qRT-PCR of *Txnip* and *Ddit3* in KP cells expressing two independent *Yap1* shRNAs. Error bars represent SD.

*Shaui Ye, Susan Chor, and Jaimarie Sostre-Colón helped by performing experiments and analyzing data.

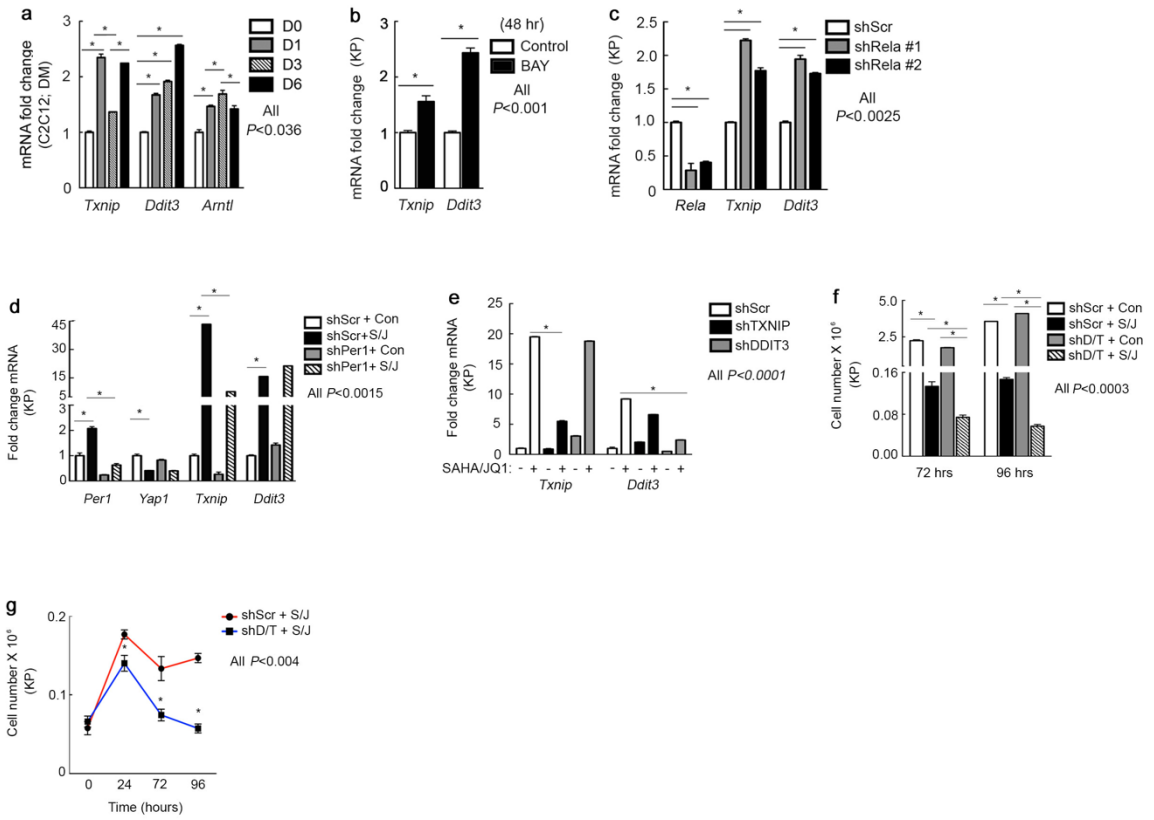


Figure 12: *Txnip* and *Ddit3* are necessary for survival during muscle differentiation

A) qRT-PCR for *Txnip* and *Ddit3* genes in proliferating (Day 0, D0) and differentiating (D1-D6) C2C12 myoblasts.
B) qRT-PCR of KP cells treated with NF-κB inhibitor 1.5 μM BAY 11-7085 for 48 hrs.
C) qRT-PCR of *Txnip* and *Ddit3* in KP cells expressing *Rela* shRNAs.
D) qRT-PCR rescue assay of KP cells expressing *Per1* shRNA and treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
E) qRT-PCR of KP cells expressing *Txnip* or *Ddit3* shRNAs treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
F) Cell counting rescue proliferation assay in cells expressing both *Txnip* and *Ddit3* shRNAs and treated as in A. D/T shRNAs denotes *Ddit3* and *Txnip* shRNAs were used for knockdown.
G) Cell counting proliferation assay of KP cells from M from 0-96 hrs. Error bars represent SD.

*Gabrielle E. Ciotti and Susan Chor helped by performing experiments and analyzing data.

SAHA/JQ1 treatment promotes oscillation of UPR target genes

To further explore the link between the UPR and circadian oscillation we characterized the expression pattern of *TXNIP* and *DDIT3* in both UPS and fibrosarcoma cells. *TXNIP* mRNA expression exhibited a dramatic circadian-like oscillation pattern with high amplitude changes in KP and HT-1080 cells over 0-120hrs of treatment with SAHA/JQ1, whereas the amplitude of *DDIT3* oscillations were more limited (**Figure 13a-d)**. Importantly, these substantial oscillations were only found in cells treated with the drug combination, whereas individual treatments had minimal effects on *Txnip* and *Ddit3* levels. YAP1 levels did not oscillate above 1 under these conditions but were decreased over time (**Figure 13d)**. Thus, we report that that the UPR targets *Txnip* and *Ddit3* exhibit a circadian-like oscillation expression pattern, though not to say extent, further linking UPR to the clock in our system.

To validate that general ATF4 and ATF6-mediated transcription were upregulated in response to SAHA/JQ1 we treated KP cells expressing GFP reporters for these transcription factors for 24 hrs and found substantial activation of both ATF4 and ATF6-mediated transcription (**Figure 14 and b**). To demonstrate that YAP1 suppresses UPR *in vivo* in our system we stained tumor sections from allografts expressing scramble shRNA vs Rela shRNA as well as our KP, KPY, and SAHA/JQ1 treated KP tumors for Gadd34 expression. We found that loss of Yap1/NF-κB upregulated Gadd34 expression *in vivo* (**Figure 14c and d**). Next, we performed IHC to investigate TXNIP and CHOP expression in human skeletal muscle compared to UPS and observed decreased nuclear staining in UPS relative to muscle tissue (**Figure 14e**).

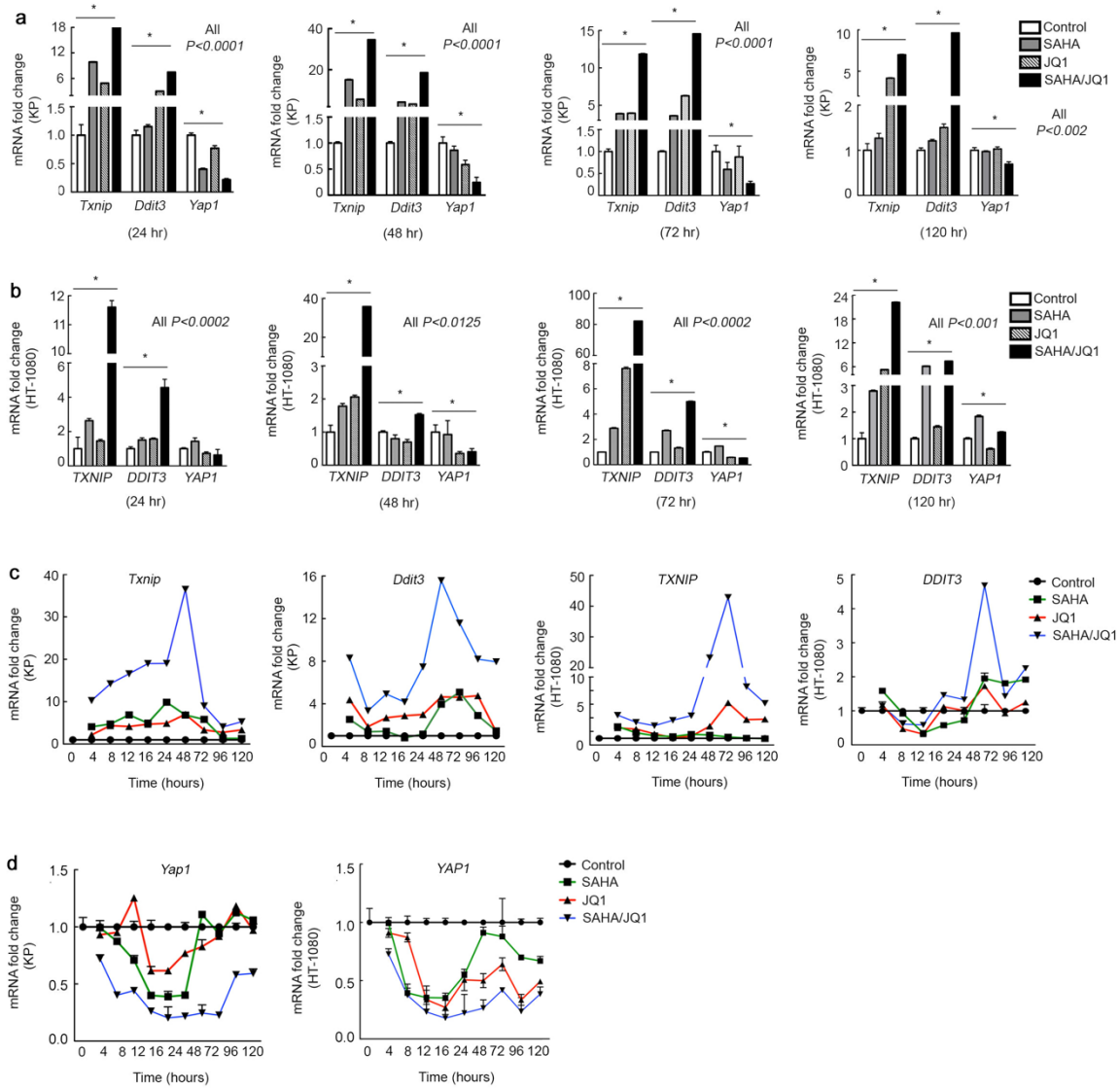


Figure 13: SAHA/JQ1 treatment promotes UPR target oscillation

A) qRT-PCR of KP cells treated with 2 μM SAHA and/or 0.5 μM JQ1 for 0-120 hrs.
B) qRT-PCR of HT-1080 cells treated with 2 μM SAHA and/or 0.5 μM JQ1.
C) Summary graphs of qRT-PCR for *TXNIP* and *DDIT3* treated as in A and B with the addition of 0-24 hr time points.
D) qRT-PCR of KP (left) and HT-1080 cells (right) treated with 2 μM SAHA/ 0.5 μM JQ1 for 0-120 hrs.

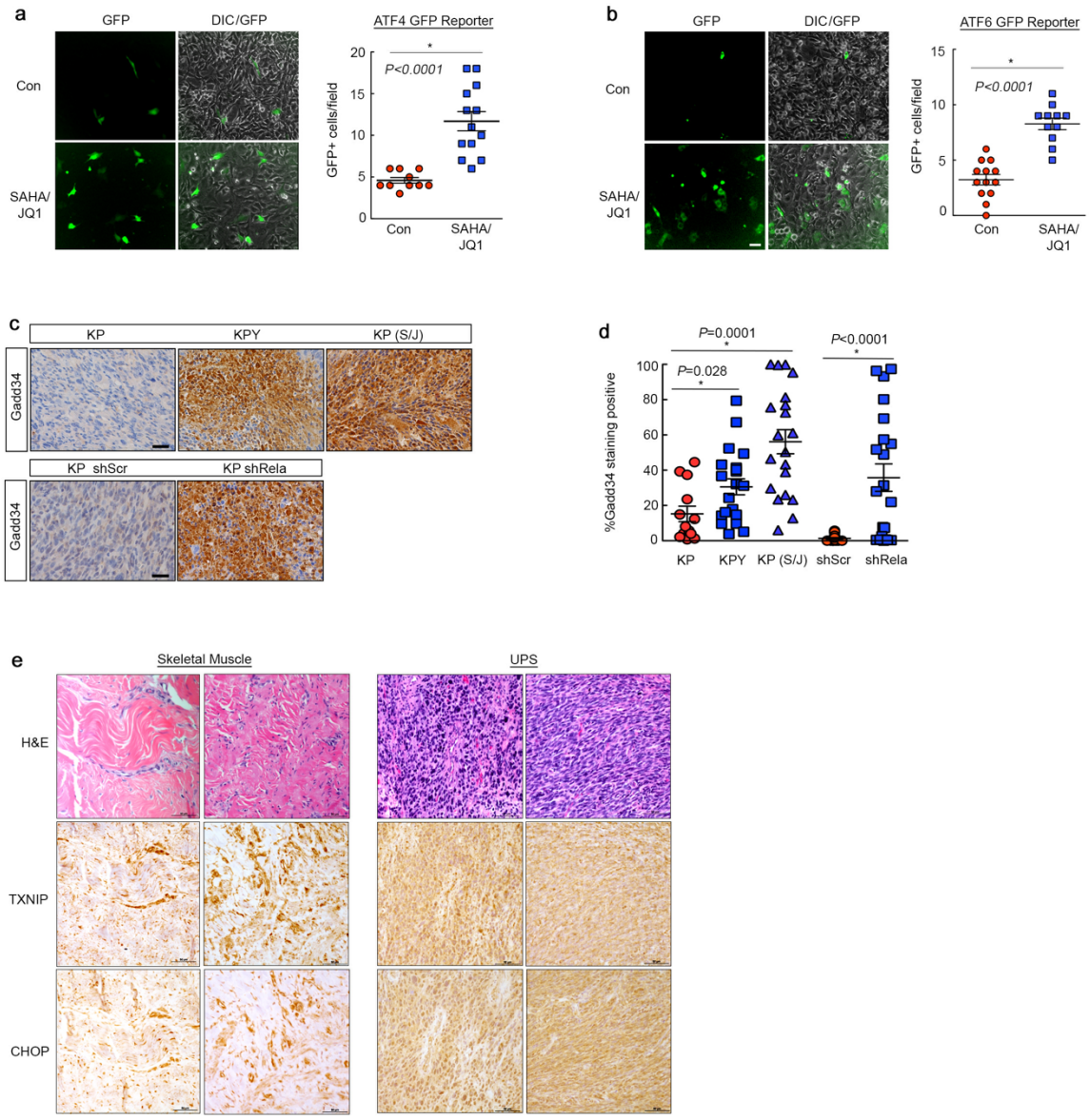


Figure 14: SAHA/JQ1 treatment induces the PERK and ATF6 arms of the UPR

A) ATF4-GFP reporter assay in KP cells treated as in A for 24 hrs. Scale bar= 50 μm.
B) ATF6-GFP reporter assay in KP cells treated as in A for 24 hrs. Scale bar= 50 μm.
C) Representative images of IHC from murine tumor sections from allograft experiments (shSCR, shRelA) and GEMMs (KP, KPY, and KP tumors treated with SAHA/JQ1 once daily with 25mg/kg SAHA and twice daily with 25mg/kg JQ1). Tumors harvested after 20 days of treatment. Scale bar= 20 μm.
D) G) Quantification of Gadd34 expression from F. n=3 mice per group, 12 images per tumor sample. Error bars represent SD.
E) Representative IHC of normal TXNIP and CHOP expression in human skeletal muscle and UPS tissues. Scale bar= 50 μm.

*Jessica M. Posimo, Gabrielle E. Ciotti, and Susan Chor helped by performing experiments and analyzing data.

Pharmacological and genetic inhibition of YAP1 alters sarcoma cell metabolism

The primary known function of circadian circuitry is to regulate cellular metabolism (Bass & Takahashi 2010; Altman et al. 2015). Cancer cell metabolism is highly deregulated and favors the rapid, yet inefficient, energy production associated with glycolysis (Altman et al. 2015). We hypothesized that activation of clock gene expression would alter the metabolic phenotype in sarcoma cells. Therefore, we evaluated key metabolic genes in our microarray analysis of DMSO and SAHA/JQ1 treated KP cells and observed differential gene expression of cancer metabolism hallmarks, such as Fatty acid synthase (*Fasn)* (**Figure 15a**). We also evaluated expression of the hallmarks of normal metabolism in differentiated cells and tissues, *Cpt1a* and *Cpt1b. CPT1A and CPT1B* are associated with β-oxidation of fatty acids, a slower but more efficient metabolic process (Carracedo et al. 2013). CPT1B is the isoform generally associated with metabolism of differentiated muscle, whereas *CPT1A* can induce autophagy (Niso-Santano et al. 2015). We found that *Fasn* was downregulated dramatically along with *Yap1,* while *Cpt1a and Cpt1b* and the muscle differentiation factor *Mef2c* were elevated in SAHA/JQ1 treated cells. Interestingly, *Cpt1a* was increased substantially more than *Cpt1b.* We then evaluated publicly available datasets for expression of *CPT1A*, *CPT1B, and FASN* in skeletal muscle, additional normal human tissues, fibrosarcomas, and UPS. *CPT1A* levels in most of these tumors are modestly elevated or unchanged relative to normal tissues (**Figure 15b**) whereas muscle-specific *CPT1B* is lost and *FASN* is increased uniformly in fibrosarcomas and UPS. (**Figure 15c and d**). We observed that SAHA/JQ1 treatment suppressed *Fasn* expression and upregulated *Cpt1a* by qRT-PCR (**Figure 15e and f**) and Western blot of murine and human UPS cells (**Figure 15g and h**). These data suggest that *Cpt1a* induction may compensate for the lack of substantial *Cpt1b* increase during SAHA/JQ1-mediated differentiation of KP cells (**Figure 15a**). Consistent with our prediction that metabolic changes are linked to differentiation, Fasn is substantially decreased in our C2C12 differentiation model by day 6 (**Figure 15i**). To determine if these metabolic alterations were directly linked to the Yap1/NF-κB axis we silenced *Yap1* (**Figure 15j, left**) or *Rela* (**Figure 15j, right**) in KP cells with specific shRNA. Inhibition of *Yap1* or *Rela* increased *Cpt1a* and *Mef2c*

mRNA expression (**Figure 15j**), suggesting that YAP1 is necessary to suppress differentiation, potentially via β-oxidation of fatty acids or autophagy mediated by the CPT1 enzymes. Next, we performed a rescue experiment to determine if YAP1-mediated suppression of the circadian clock controls metabolism and differentiation. We treated KP cells with SAHA/JQ1 and *Per1* shRNA alone or in combination. We observed an increase in *Cpt1a* and *Mef2*c and loss of *Yap1* under SAHA/JQ1 treatment as predicted (**Figure 15k**). Moreover, loss of Per1 prevented SAHA/JQ1-mediated induction of *Cpt1a* and *Mef2c*. We observed the same trends in KP cells treated with SAHA/JQ1 and *Arntl*-specific shRNA (**Figure 15l**). Interestingly, *Fasn* gene expression appears to be YAP1-independent. Our shRNA-mediated YAP1 inhibition shows no reduction of *Fasn* (not shown) and loss of clock activity does not restore *Fasn* levels. These observations suggest that YAP1 signaling may coordinate with at least one other pathway to modulate metabolism. To further characterize the metabolic status of control vs. SAHA/JQ1-treated KP cells we performed GSEA of the DMSO vs. SAHA/JQ1 microarray. We observed that "MTORC1 signaling" is downregulated in treated cells (**Figure 15m**), which is consistent with the observations of several other groups who reported that YAP1 regulates this pathway (Hu et al. 2017; Park et al. 2016). MTORC1 promotes cell growth by activating biosynthesis and suppressing autophagy (Jung et al. 2010; Laplante & Sabatini 2012). The GSEA also revealed that "Glycolysis" is significantly downregulated in SAHA/JQ1 treated cells, while "lipid catabolic processes" are upregulated. Together, these data show that the Yap1/NF-κB axis favors the expression of cancer associated metabolism markers and that inhibition of this pathway promotes expression of muscle markers and muscle-associated metabolism.

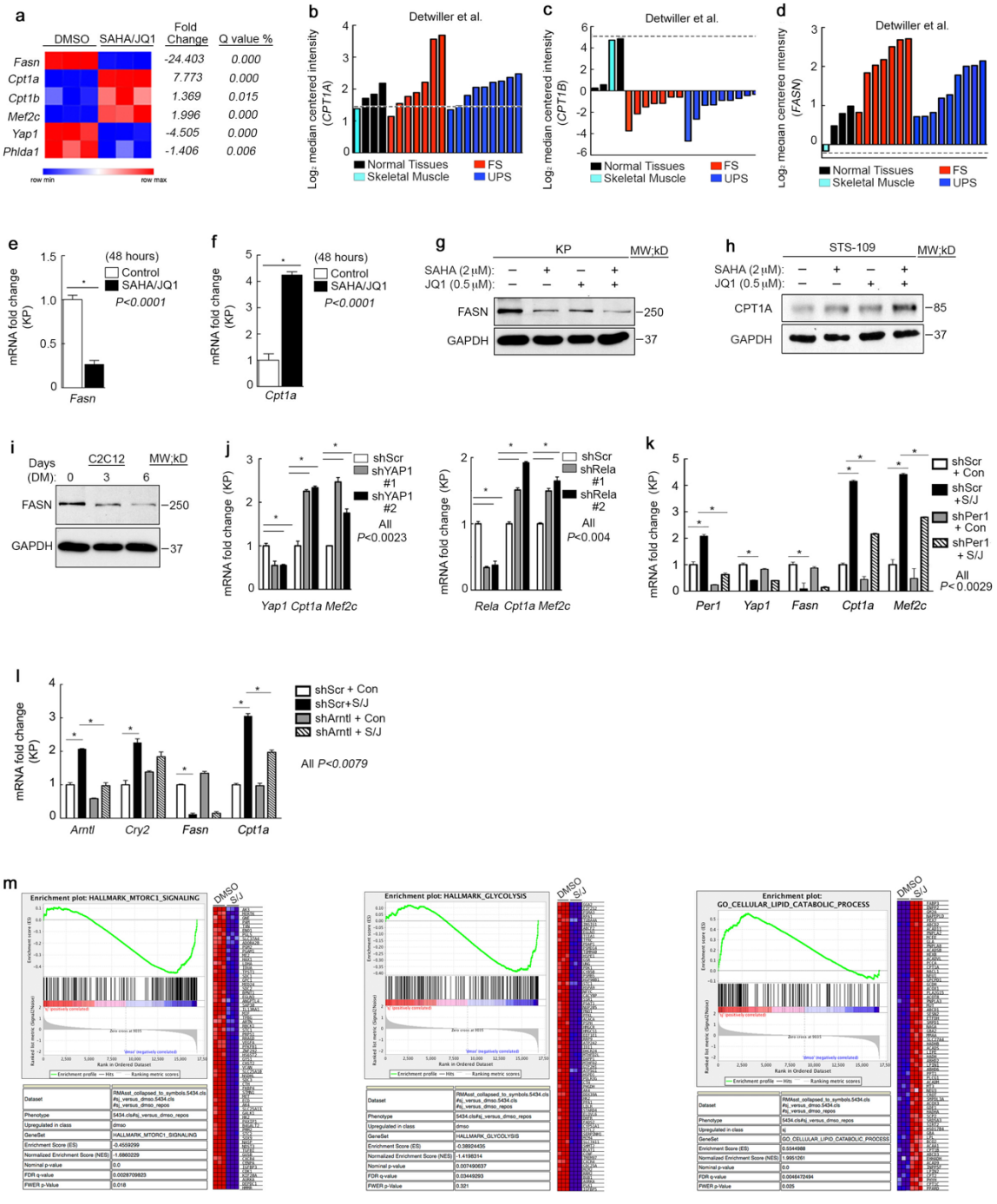


Figure 15: YAP1 loss alters sarcoma cell metabolism and initiates differentiation

A) Gene expression analysis of microarray performed on KP cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
B) Oncomine gene expression analysis of *CPT1A* in human tissues.
C) Oncomine gene expression analysis of *CPT1B* in human tissues.

D) Oncomine gene expression analysis of *FASN* in human tissues.
E) qRT-PCR validation of *Fasn* in KP cells treated as in A.
F) qRT-PCR validation of *Cpt1a* in KP cells treated as in A.
G) Western blot of KP cells treated as in A.
H) Western blot of STS-109 cells treated as in A.
I) Western blot of proliferating (Day 0, D0) and differentiating (D1-D6) C2C12 myoblasts.
J) qRT-PCR of KP cells expressing (right) Yap1 shRNAs and (left) Rela shRNAs.
K) qRT-PCR rescue assay of KP cells expressing *Per1* shRNA and treated as in A.
L) qRT-PCR of KP cells expressing Arntl shRNA and treated as in A.
M) GSEA analysis of microarray from KP cells treated as in A using the Broad Institute "hallmark" gene sets for "MTORC1 signaling", "Glycolysis", and "Lipid catabolic process". Error bars represent SD.

*Shuai Ye, Gabrielle E. Ciotti, Susan Chor, Gloria Marino, and Shaun Egolf helped by performing experiments and analyzing data.

YAP1, but not NF-κB, suppresses autophagy in UPS cells

In addition to its established role in fatty acid oxidation, CPT1A is also associated with upregulation of autophagy (Niso-Santano et al. 2015). To determine which of these two processes occurs during SAHA/JQ1-mediated differentiation we performed GCMS evaluating oxidation of 75 μM [U-$^{13}C_{16}$]palmitic acid and 75 μM [U-$^{13}C_{18}$]oleic acid via enrichment of TCA cycle intermediates and observed a modest but reproducible decrease in β-oxidation of fatty acids in SAHA/JQ1 treated cells (**Figure 16a**). These data suggest that β-oxidation changes do not correlate with the differentiation phenotype induced by epigenetic modulation. Therefore, we investigated the ability of this drug combination to induce autophagy. Autophagy is directly associated with muscle function and maintenance of muscle mass (Fortini et al. 2016; Masiero et al. 2009). The DMSO vs. SAHA/JQ1 microarray revealed that treatment upregulates many genes associated with autophagy including *Atg13* and *Atg14,* which are specifically linked to muscle development and function (**Figure 16b**). Importantly, we also saw dramatic induction of the autophagy marker LC3 in KP cells treated with SAHA/JQ1 and Bafilomycin (BAF) (**Figure 16c, top**). BAF interrupts autophagic flux by inhibiting maturation of autophagic vacuoles during the late stages of autophagy, forcing accumulation of the autophagosome-associated LC3-II. Consistently, shRNA-mediated depletion of Yap1 also enhanced LC3A/B expression in BAF treated cells, relative to control shRNA (**Figure 16c, bottom**). Using *Yap1* shRNA we also screened KP cells to determine which autophagy related genes are Yap1-dependent and observed that Yap1 loss upregulates *Atg13* and *Atg14* mRNA expression (**Figure 16d**). Additionally, we observed increased *Atg14* mRNA expression in tumors from KPY tumors relative to KP (**Figure 16e**). Most significantly, we observed loss of p62 and dramatic accumulation of LC3B *in vivo* in KPY (Yap1-deleted) tumors and KP tumors treated with SAHA/JQ1 relative to control KP tumors (**Figure 16f-h**). SAHA/JQ1 treatment was initiated when KP tumors reached 100mm^3 according to the schedule found in **Figure 16i**. Together these findings clearly link YAP1 to autophagy regulation in UPS. Lastly, we investigated whether YAP1-mediated suppression of autophagy was NF-κB- and clock-dependent. Inhibition of NF-κB using Rela shRNA had no effect

on LC3A/B accumulation (**Figure 16j**) or *Atg13* and *Atg14* levels (**Figure 16k**). Moreover, we expressed *Per1* shRNA in SAHA/JQ1 treated KP cells and found that clock modulation has no effect on *Atg13* and *Atg14* induction (**Figure 16l**) and that Atg13 does not oscillate at all during C2C12 differentiation, whereas Atg14 oscillates extremely modestly (**Figure 16m)**. These data indicate that *ATG13* and *ATG14*, and perhaps autophagy in general is not required for muscle differentiation but is required for muscle maintenance and function as demonstrated by other groups (Masiero et al. 2009; Sandri et al. 2013). Lastly, we investigated potential crosstalk between the UPR and autophagy in this context. We treated KP cells expressing Txnip/Ddit3 specific shRNAs with SAHA/JQ1 and BAF. Inhibition of Txnip and Ddit3 had no effect on SAHA/JQ1-mediated autophagy (**Figure 16n)**. We conclude from these observations that YAP1 suppresses autophagy. These data reveal that SAHA/JQ1 induces autophagy via YAP1 inhibition in sarcoma cells. Moreover, my data shows that induction of autophagy in sarcoma cells is independent of NF-κB signaling indicating that YAP1 controls multiple key aspects of cell survival and proliferation in muscle-derived sarcoma cells (**Figure 17a and b**).

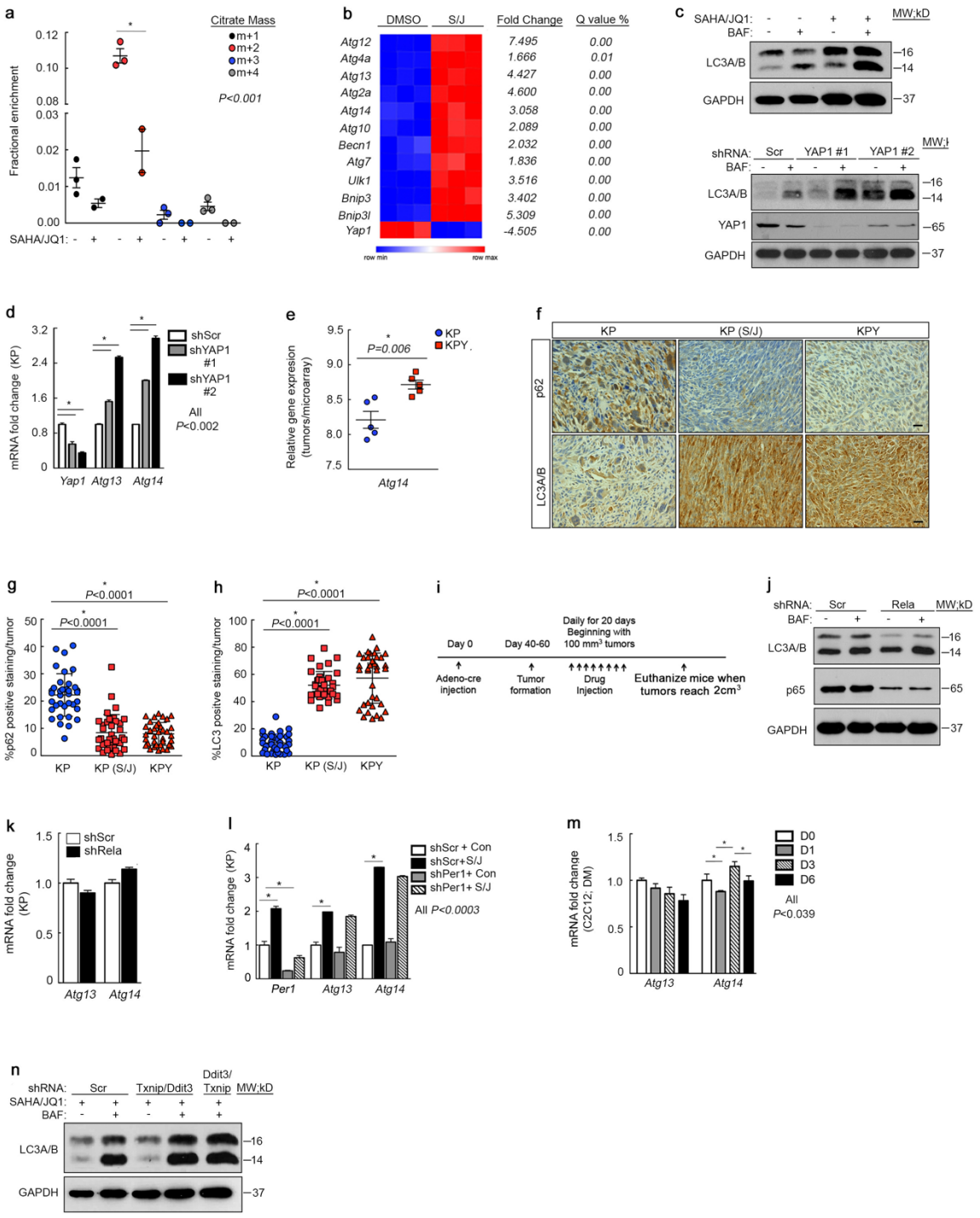


Figure 16: YAP1 suppresses autophagy in sarcoma cells independent of NF-κB

A) GC/MS of KP cells treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
B) Gene expression analysis of microarray described in A.

C) (top) Western blot of KP cells treated as in A with the addition of BAF during the last 6 hr of treatment. (bottom) Western blot of YAP1 shRNA expressing cells treated with BAF as in the top panel.
D) qRT-PCR of *Atg13* and *Atg14* in KP cells expressing *Yap1* shRNA.
E) qRT-PCR of KP and KPY tumors.
F) Representative images of IHC from murine tumor sections from KP, KPY, and KP tumors treated with SAHA/JQ1 once daily with 25mg/kg SAHA and twice daily with 25mg/kg JQ1. Tumors harvested after 20 days of treatment. Scale bar= 20 μm.
G) Quantification of p62 expression from F. n=3 mice per group, 12 images per tumor sample.
H) Quantification of LC3B expression from F n=3 mice per group, 12 images per tumor sample.
I) Drug scheduling for KP GEMM treatment beginning when tumors measure 100mm^3.
J) Western blot of KP cells expressing Scr or Rela shRNAs treated as in A with the addition of BAF during the last 6 hrs of treatment.
K) qRT-PCR of KP cells expressing Rela shRNA.
L) J) qRT-PCR rescue assay of KP cells expressing *Per1* shRNA and treated with 2 μM SAHA/ 0.5 μM JQ1 for 48 hrs.
M) qRT-PCR for *Atg13* and *Atg14* genes in proliferating (Day 0, D0) and differentiating (D1-D6) C2C12 myoblasts.
N) Western blot of KP cells expressing both *Txnip* and *Ddit3* shRNAs and treated as in B with the addition of BAF during the last 6 hrs of treatment. *Txnip/Ddit3* denotes that KP cells were first transfected with *Txnip* shRNA followed by *Ddit3* shRNA, and inverse for *Ddit3/Txnip* lane. Error bars represent SD.

*Shuai Ye, Gabrielle E. Ciotti, Susan Chor, Ying Liu, Gloria Marino, and Shaun Egolf helped by performing experiments and analyzing data.

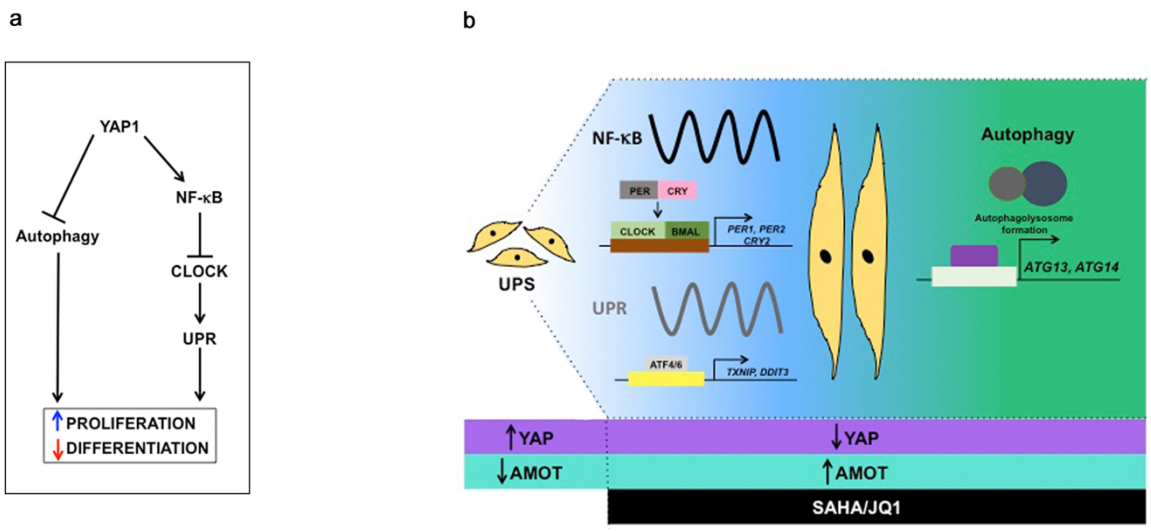


Figure 17: Model of YAP1/NF-κB-mediated clock control of UPR and NF-κB-independent control of autophagy

Discussion

Though well studied in epithelial tumors, the specific downstream effectors of YAP1 in sarcomas are still being elucidated. Characterization of these effectors is necessary for the development of effective biomarkers and targeted therapies to treat YAP1-dependent tumors including UPS. Previously, we demonstrated that YAP1 promotes proliferation via persistent elevated NF-κB signaling (Ye et al. 2018). The goal of this study was to elucidate the mechanisms by which YAP1 and NF-κB impact muscle de-differentiation and promote tumorigenesis. My work shows that YAP1-mediated NF-κB signaling represses UPR and circadian clock activity, both of which are required for muscle differentiation (Andrews et al. 2010; Xiong et al. 2017). We also show that YAP1 inhibits autophagy in an NF-κB-independent manner.

To our knowledge, this work provides the first direct link between the Hippo pathway and circadian clock activity. Using our autochthonous mouse models, I demonstrated that Yap1-mediated NF-κB activity disrupts normal circadian oscillation by suppressing *Per1, Per2* and *Cry2* levels. Consistent with this observation, *PER1, PER2,* and *CRY2* are downregulated in human UPS (Ye et al. 2018). We also demonstrate that genetic or pharmacological inhibition of YAP1 enhances circadian clock activity and the oscillation of key targets including *Txnip,* which is a major effector of the UPR (Oslowski et al. 2012; Y. Ma et al. 2002).

One critical purpose of the UPR and circadian clock is regulation of metabolic processes (Bohnert et al. 2018). We found that Yap1-mediated suppression of the UPR and clock support a shift in metabolism toward cancer cell-associated glycolysis and hyper-proliferation. Reactivation of the UPR and clock via SAHA/JQ1 correlate with decreased glycolysis while enhancing autophagy and lipid catabolism, thus promoting skeletal muscle differentiation.

Our work sheds light on several on-going areas of cancer research. First, our observation that the clock and the UPR are both activated in differentiating myoblasts, as well as SAHA/JQ1-

treated sarcoma cells, indicates that the relationship between these two processes is context-dependent. Whereas in some tissues the UPR antagonizes clock gene expression (Bu et al. 2018), in skeletal muscle and muscle-derived tumors both pathways are critical for differentiation and can be simultaneously upregulated (Andrews et al. 2010; Xiong et al. 2017; Bohnert et al. 2016). This idea is consistent with several studies showing that UPR is necessary for muscle regeneration and maintenance of muscle mass (Bohnert et al. 2018; Xiong et al. 2017; Bohnert et al. 2016; Nakanishi et al. 2007).

Similarly, clock function is necessary for muscle generation and differentiation. In fact, MyoD, is a direct transcriptional target of the molecular clock (Andrews et al. 2010; X. Zhang et al. 2012; Lefta et al. 2011). Clock deficient mice suffer from muscle weakness, cachexia, and disrupted metabolism (Bohnert et al. 2016). However, little is known about upstream signaling inputs that control clock gene expression and function. Now we appreciate that aberrant Yap1 stabilization impacts these processes in muscle-derived sarcomas and potentially other contexts as well. In future studies, we will determine how Yap1 suppresses clock, UPR gene expression, and autophagy. Yap1 is generally considered a transcriptional activator. As such, our findings highlight novel roles for Yap1 in suppressing transcription. The targets repressed by Yap1 are particularly intriguing to us. Whereas, we might predict that Yap1 would inhibit expression of pro-apoptotic genes our work suggests that Yap1, via NF-κB, also represses pro-differentiation genes. This hypothesis is supported by data indicating that Yap1 inhibits muscle differentiation in C2C12 myoblasts (Watt et al. 2010).

Lastly, we sought to determine the utility of epigenetic modulation in the treatment of muscle-derived sarcomas. We previously reported their impressive efficacy in the KP GEMM (Ye et al. 2018). Here we validate our earlier finding that this strategy inhibits proliferation and enhances differentiation. We have identified key differentiation targets including clock, UPR, and

autophagy genes as biomarkers of SAHA/JQ1 efficacy and suggest that this therapeutic strategy and these markers may offer clinical benefit to some sarcoma patients.

Chapter 4: Conclusion

Our results identify a network of signaling mechanisms regulated by Hippo signaling pathway inactivation and YAP1 stabilization to promote sarcomagenesis. Although YAP1 has been extensively studied in epithelial tumors, the downstream effectors of YAP1 in sarcoma remain to be elucidated. Thus, characterization of these effectors is of high importance for the development of targeted therapies and as effective biomarkers in patients with UPS and other YAP1-dependent tumors. We have previously demonstrated that YAP1 hyperactivation and stabilization promotes persistent canonical NF-κB signaling. Activation of the YAP1-NF-κB axis promotes UPS proliferation and sarcomagenesis, and antagonizes muscle differentiation (Ye et al. 2018). Thus, it was our goal to elucidate the downstream mechanisms by which the YAP1-NF-κB axis promotes sarcomagenesis and inhibits muscle differentiation.

My work demonstrates that inactivation of Hippo signaling pathway, resulting in YAP1 stabilization, inhibits muscle differentiation and promotes proliferation and sarcomagenesis in UPS by repressing circadian clock activity, the UPR, and autophagy. Consistent with the observation that *PER1*, *PER2*, and *CRY2* are downregulated in human UPS, I show that the YAP1-NF-κB axis suppresses *Per1*, *Per2*, and *Cry2* levels *in vivo* and that genetic and pharmacological inhibition of YAP1 reactivates circadian clock activity *in vitro*. Moreover, genetic and pharmacological ablation of YAP1 enhanced oscillation of the circadian clock genes and *Txnip,*, a major effector of the UPR (Bohnert et al. 2016; Nakanishi et al. 2007). Activation of the UPR and circadian clock become activated in differentiating myoblasts and SAHA/JQ1 treated sarcoma cells further demonstrates that the relationship between these two processes is context dependent. In summary, these findings shed light on multiple on-going areas of cancer research.

Though in some cancers and tissues the UPR has been shown to antagonize clock gene expression, in skeletal muscle and muscle-derived tumors activation of the UPR and circadian

clock are important for differentiation and survival during differentiation (Bu et al. 2018). In UPS we found that YAP1 suppresses the UPR and circadian clock, thus both pathways can be simultaneously upregulated (Andrews et al. 2010; Xiong et al. 2017; Bohnert et al. 2016). This is consistent with the idea that the UPR is necessary for skeletal muscle regeneration and muscle mass maintenance (Bohnert et al. 2018; Xiong et al. 2017; Bohnert et al. 2016). Moreover, the main muscle specific transcription factor, MyoD, is a direct transcriptional target of the circadian clock (Andrews et al. 2010; X. Zhang et al. 2012; Lefta et al. 2011). Additionally, ER stress and the UPR target protein CHOP and the ATF6 arm of the UPR are also known to play a role in muscle differentiation and myofiber formation (Alter & Bengal 2011; Nakanishi et al. 2005; Nakanishi et al. 2007). My work suggests that YAP1, via NF-κB, represses the UPR, circadian clock, and pro-differentiation genes. This hypothesis is supported by data indicating that YAP1 inhibits muscle differentiation in C2C12 myoblasts (Watt et al. 2010).

The circadian clock and UPR are also known to regulate metabolic processes (Bohnert et al., 2018). We found that inhibition of both the UPR and circadian clock supports a shift in metabolism toward cancer cell-associated glycolysis and hyperproliferation. In contrast, reactivation of the UPR and circadian clock promoted a shift from cancer cell-associated glycolysis and hyperproliferation to lipid catabolism and enhanced autophagy. Whereas autophagy is necessary for skeletal muscle differentiation and survival during differentiation, clock deficient mice suffer from muscle weakness, cachexia and disrupted metabolism (Andrews et al. 2010; Bohnert et al. 2016). These observations further support our findings that deregulation of circadian clock, UPR, and autophagy gene expression and activity inhibit skeletal muscle differentiation and promotes proliferation and growth. Furthermore, my work demonstrates that YAP1-mediated inhibition of autophagy is independent of NF-κB signaling. In terms of the upstream signals that control the clock, little is known.

Finally, we sought to determine the effects of epigenetic drugs as potential treatments for muscle-derived sarcomas. We validated the use of SAHA/JQ1 as a treatment modality in our in vivo and in vitro system. Similar to genetic ablation of YAP1, treatment with SAHA/JQ1 induces differentiation and inhibits proliferation. Additionally, SAHA/JQ1-mediated activation of the UPR enhances autophagy. My work identified key UPR, circadian clock and autophagy differentiation targets that could function as biomarkers of SAHA/JQ1 efficacy and could offer clinical benefits to some sarcoma patients.

Future Directions

YAP1 as a transcriptional repressor

YAP1 is a known potent transcriptional regulator. Our data highlight novel roles for Yap1 in suppressing transcription. In future studies, we will determine the mechanism by which Yap1 suppresses circadian clock, UPR, and autophagy gene expression and activity. As discussed previously, YAP1 must translocate into the nucleus and bind the Tea Domain Family of transcription factors (TEAD) proteins to promote a pro-proliferation transcriptional program (Ye et al. 2018; Liu-Chittenden et al. 2012; Zhao et al. 2007; Barron & Kagey 2014). We have previously shown that YAP1 predominantly interacts with TEAD1 and TEAD4 in sarcoma cells (Eisinger-Mathason et al. 2015). My work, however, has shed light on the paradigm shifting concept, proposed by us, that YAP1 can potentially function as a transcriptional repressor of UPR, circadian clock, and autophagy genes in order to impair muscle differentiation.

To determine that YAP1 is directly inhibiting transcription of UPR, circadian clock, and autophagy genes we will perform ChIP-seq and RNA-seq. We will first validate if TEAD binding sites exist proximal to the various target genes affected by YAP1 and described in Chapter 3. I expect the YAP1-TEAD complex to bind the M-CAT motif and inhibit transcription of target genes. Moreover, we will determine the epigenetic landscape of the target genes, which I expect to be in inaccessible to factors that promote transcription. Alternatively, it is possible that YAP1-TEAD

promotes transcription of genes or non-coding RNAs that repress transcription of UPR, circadian clock, and autophagy genes.

Mouse Models and Pharmacological Approaches

To further test the contribution of NF-κB signaling to sarcomagenesis *in vivo*, we developed the KPR mouse model. We observed that KPR mice do not develop tumors. Moreover, we validated this finding by performing genetic knockdown of RelA in our *in vitro* system and reduced sarcomagenesis as well as increased circadian clock gene transcription and activity, and UPR gene transcription. Thus, NF-κB ablation led to reduced sarcoma growth/proliferation. To determine if constitutive activation of NF-κB rescues YAP1 deficient proliferation, we will breed KPY mice (YAP deficient) with LSL-Ikk2^{CA} to generate KPYI^{CA} mice. I expect that KPYI^{CA} tumor size will be comparable to KP tumors, indicating that constitutive NF-κB activity rescues the loss of Yap1 in this context. Furthermore, we will treat KPYI^{CA} mice with SAHA/JQ1 to determine if we can extend animal survival, which would further support a model in which SAHA/JQ1 inhibits NF-κB activity and promote a potential shift from a pro-proliferation transcriptional program to a differentiation transcriptional program. Alternatively, our KP versus KPY microarray analysis revealed a list of NF-κB targets that includes *Phlda1, Bcl3, Areg, and Egr1*. If the current shortlist of NF-κB targets are not individually necessary for UPS proliferation, we will explore combination deletions as well additional targets identified by gene expression analysis in SAHA/JQ1 treated cells and YAP1 deficient cells. Then we will determine the whether these targets are critical for differentiation rather than proliferation, using a C2C12 model of undifferentiated myoblasts.

My work shows that YAP1-NF-κB axis inhibits UPR and circadian clock gene expression and activity. Moreover, I show that YAP1 inhibits autophagy in an NF-κB independent manner. Thus, it is of great interest for us to test the contribution of the UPR, circadian clock, and

autophagy in UPS initiation, progression and survival. To determine the contributions of the circadian clock in UPS, we will cross KPY mice with BMAL deficient mice to develop KPYB. To test the contribution of the UPR, we will cross KPY mice with TXNIP and CHOP to develop KPYT and KPYC mice. I expect KPYB, KPYT and KPYC tumors to be larger in size than KPY tumors but smaller than KP tumors, as our findings suggest that circadian clock and UPR activity promotes muscle differentiation and inhibits UPS proliferation. Last, to determine the contribution of autophagy in UPS we will cross KPY mice with FIP200 conditional knockout mice to produce KPYF. FIP200 is a component of a molecular complex that includes ATG13, required for autophagosome formation, and known to promote mammary tumorigenesis ((Hara & Mizushima 2009; Wei et al. 2011). I expect that KPYF tumor size will be smaller than KPY tumors, indicating that autophagy is required for UPS survival. Alternatively, we can treat KPY mice with known pharmacological inhibitors of the UPR and autophagy, such as 4-PBA and chloroquine, respectively. Alternatively, to inhibit the clock, we would follow a new approach, established in Chi Dang's lab, in which stabilizing HIF with DMOG and/or increasing the acidity of media disrupts circadian clock oscillation (Walton et al. 2018).

Future potential treatments for sarcoma

Our data show the Hippo pathway inhibits the UPR, circadian clock and autophagy, which all contribute and are important for muscle differentiation. Currently there are no targeted therapies for soft tissue sarcoma patients (M. Zhang et al. 2014). However, we observed inactivation of the Hippo pathway and increased stabilization of YAP1 in UPS (Eisinger-Mathason et al. 2015). Thus, YAP1 drives a pro-proliferation transcriptional program that promotes sarcomagenesis and metastasis (Eisinger-Mathason et al. 2015). In UPS, we observed that YAP1 stabilization and translocation into the nucleus occurs because of epigenetic silencing of its inhibitor, AMOT, and Hippo pathway kinase deletion (Ye & Eisinger-Mathason 2016). Additionally, in human UPS tumor sections, we observed increase protein expression of the canonical NF-κB transcriptional factor, phosphorylated p65. We demonstrated that YAP1, in fact, promotes

sarcomagenesis and inhibits muscle differentiation, in part, by sustaining constitutive canonical NF-κB signaling (Ye et al. 2018). Moreover, we have demonstrated that KP cells are sensitive to the NF-κB inhibitors BAY11-7085 and CAPE (Ye et al. 2018). Thus, NF-κB inhibition offers new and viable therapeutic options to treat sarcoma. It is important to note that the most widely used NF-κB inhibitors available have specificity issues. Thus, we are currently working in collaboration with Dr. Anne Carpenter's laboratory to develop our own NF-κB inhibitor. It is our goal to develop a specific NF-kB pharmacological inhibitor.

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